MULTIPLE FORMS OF γ-GLUTAMYLTRANSFERASE

by

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DECLARATION OF ORIGINALITY

I declare that the work presented herein and the composition of this thesis is my own.

Philip R. Wenham

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Abstract

The physical properties of the multiple forms of γ-glutamyltransferase in human bile and serum have been studied and compared with those of the enzyme present in human liver. Leucine aminopeptidase and alkaline phosphatase were included, where appropriate, in the study to see whether the findings were applicable on a wider basis. γ-Glutamyltransferase is present in human bile and serum mainly as hydrophobic forms, together with small amounts of a low molecular mass hydrophilic form. The hydrophobic enzyme in serum is of both high and intermediate molecular mass, whereas in bile it is of high molecular mass only. In the presence of an adequate concentration of bile salts, the hydrophobic forms in liver, bile and serum are converted to a low molecular mass, hydrophobic form which reaggregates in the absence of bile salts. When treated with papain, the hydrophobic form of the enzyme in liver, bile and serum is converted to a low molecular mass hydrophilic form with identical physical properties to the hydrophilic form present in small amounts in native bile and serum. Detailed study of the γ-glutamyltransferase fraction of intermediate molecular mass in serum from patients with a variety of liver diseases showed it to consist of a complex between γ-glutamyltransferase and high density lipoprotein.

In a clinical study it was shown that the amount of high molecular mass γ-glutamyltransferase in serum, particularly when expressed as a percentage of the total γ-glutamyltransferase activity, was raised to a greater extent in patients with extrahepatic biliary obstruction than in those with other liver diseases. It was also shown that γ-glutamyltransferase in serum could be separated into several bands of intermediate molecular mass by electrophoresis. One of these bands predominated in patients with extrahepatic biliary obstruction but not in patients with other forms of liver disease. Although either of these observations could be of value as a clinical test to distinguish extrahepatic from intrahepatic jaundice, it was concluded that the electrophoresis of serum γ-glutamyltransferase was likely to be a more effective and simpler test.

CONTENTS

Abbreviations

The abbreviations in this thesis are those used by the Biochemical Journal 1983; 209: 1 - 27.

The following abbreviations has also been used:

Saline,	150 mmol/l NaCl

Chapter 1

INTRODUCTION

γ-Glutamyltranferase (γGT, EC 2.3.2.2), first described by Dakin and Dudley (1913) as antiglyoxylase and investigated further by Woodward et al. (1935), is a dimeric glycoprotein widely distributed in many human tissues and body fluids.

1.1 GENERAL PROPERTIES OF γ-GLUTAMYLTRANSFERASE

1.1.1 Catalytic actions

γGT catalyses 3 types of reaction:

1. The transfer reaction

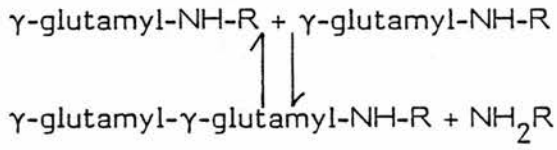
This involves the transfer of the γ-glutamyl residue from glutathione and other γ-glutamyl peptides to amino acids or small peptides, forming the γ-glutamyl-amino acid and cysteinyl-glycine:

γ-glutamyl-cysteinyl-glycine + amino acid (peptide)
⇅
γ-glutamyl-amino acid (peptide) + cysteinyl-glycine.

In most biological systems glutathione is the γ-glutamyl donor but any γ-glutamyl residue attached to a peptide, amino acid or even an artificial chromogen can serve this purpose.

2. The autotransfer reaction

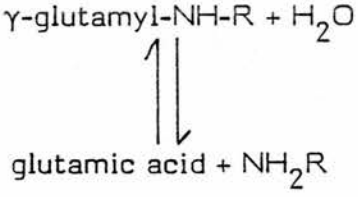
In this reaction, the γ-glutamyl residue can be transferred from one substrate molecule to another of the same substrate forming a γ-glutamyl-γ-glutamyl-peptide:



R may be an amino acid, a peptide or an artificial chromogen. The reaction is blocked by the presence of an α-methyl group attached to the γ-glutamyl residue of the substrate itself.

3. Hydrolysis

Hydrolysis occurs with the formation of free glutamic acid and either the amino acid or the peptide to which the γ-glutamyl group was initially attached:



1.1.2 Measurement of γ-glutamyltransferase activity

The earliest methods for the determination of γGT activity utilized glutathione as substrate, were only semi-quantitative and were very time consuming (Hanes et al., 1950, 1952; Ball et al., 1956; Hird and Springwell, 1954). Significant improvements came with the introduction of substrates whose product could be determined colorimetrically. Szewczuk and Orlowski (1960) used α-(N-γ-DL-glutamyl)-aminoproprionitrile, the liberated α-aminoproprionitrile being determined by reaction with bromine and coupling with benzene-pyridine. Goldbarg et al. (1960, 1963) used N-(DL-γ-glutamyl)

aniline and measured the liberated aniline by the Bratton-Marshall (1939) reaction.

Further development came with the introduction of the α-and β-isomers of γ-L-glutamyl naphthylamide as substrates (Albert et al., 196 l; Orlowski and Szewczuk, 1962; Glenner et al., 1962). Again, the Bratton-Marshall (1939) reaction was used to measure the liberated α-or β-naphthylamine, but this was later improved by direct coupling with the diazonium salt Fast Blue B (Kulhanek and Dimov, 1966).

The reactions described so far involve two-stage procedures - an enzyme reaction followed by a reaction between one of the products and a chromogen. The reaction was greatly simplified by Orlowski and Meister (1963) who used a chromogenic substrate γ-L-glutamyl-p-nitroanilide, thus eliminating the need for the second part of the reaction sequence. They were able to monitor the liberation of p-nitroaniline by measuring the rate of increase of absorbance at 410 nm. The procedure was modified by Orlowski (1965) for use as a two point assay using the autotransfer reaction described in section 1.1.1. Further improvements came with the introduction of glycylglycine as an acceptor, thus increasing the rate of reaction (Rosalki et al., 1970; Jacobs, 197 l; Beck and Sammons, 1975; Tamaoki, 1975).

The general acceptance, however, that kinetic methods are better than two point assays (Moss, 1972) led to the introduction of a kinetic assay at 25°C using this substrate (Szasz, 1969). This was later optimised at 37°C by Rosalki and Tarlow (1974) who overcame the poor solubility of the substrate by dissolving it in acid. They recommended, however, that owing to acid hydrolysis of the substrate occurring, each batch of substrate solution be used within two hours.

The problem of poor solubility of γ-L-glutamyl-p-nitroanilide was overcome by the introduction of the 3-carboxy derivative (Szasz, 1974). At present, one or other of these two substrates are now used in the vast majority of laboratories measuring serum γGT activity.

1.1.3 Enzyme mechanism

The γ-glutamyl reaction follows a ping pong bi bi mechanism (Theodorsen and Stromme, 1976; Tate et al., 1976; London, 1976; Shaw et al., 1977). The active centre consists of 3 subsites (Tate and Meister, 1981); a donor site for γ-glutamyl binding and 2 acceptor subsites, one for cysteinyl and the other for glycine moieties. Whereas the donor subsite can bind either L-or D-γ-glutamyl residues, the acceptor subsites are stereo-specific for L-amino acids and dipeptides (Tate and Meister, 1974; Thompson and Meister, 1977). Kinetic studies are consistent with the ping pong mechanism involving a γ-glutamyl-enzyme intermediate (Tate and Meister, 1974; Thompson and Meister, 1977).

$$\text{glutathione} + \text{enzyme} \rightleftharpoons \gamma\text{-glutamyl-enzyme} + \text{cysteinyl-glycine}$$

$$\gamma\text{-glutamyl-enzyme} + \text{acceptor} \rightleftharpoons \gamma\text{-glutamyl-acceptor} + \text{enzyme}$$

1.1.4 Inhibition

L-serine, in the presence of borate, has been shown to inhibit γGT from a variety of sources (Revel and Ball, 1959; Tate and Meister, 1981). This pattern of inhibition may be used as a test for the specificity of the enzyme since neither compound alone inhibits it. Serine appears to occupy the active site region that accepts the α-amino and α-carboxyl groups of glutathione, whilst the borate anion forms a bridge complex between the hydroxyl group of serine and an active site hydroxyl group (Tate and Meister, 1978). Glycine and alanine have also been shown to inhibit the enzyme (Stromme and Theodorsen, 1976).

The concentrations of these amino acids in serum are too low to influence the results of γGT measurements. However, the authors noted that alanine inhibition may present a practical problem due to contamination with alanine, if the same enzyme analyser, with automatic pipetting of the second substrate, is used to measure both γGT and alanine aminotransferase (EC 2.6.1.2, ALT).

1.2 FUNCTIONS OF γ-GLUTAMYLTRANSFERASE

1.2.1 The γ-glutamyl cycle

This cycle was first postulated and developed by Meister (1973, 1974) following his own observations and the earlier work of Hanes et al. (1950, 1952, 1954) and of Connel and Hanes (1956). According to the idea, γGT catalyses the formation of γ-glutamyl amino acids, using glutathione as γ-glutamyl donor. The γ-glutamyl amino acids are then translocated into the cell where the free amino acids are released. There then follows a series of reactions ending in the re-synthesis of glutathione which may then be used again in the cycle. This theory is consistent with the fact that γGT is localised at anatomic sites where there is substantial amino acid transport. The cycle has been shown to function in the renal tubule and is believed to be a major component of the mechanism responsible for the reabsorption of all amino acids except proline from the glomerular fitrate. It is also thought to be involved in the transport of amino acids across the blood-brain barrier and to function in all cells capable of transporting amino acids from body fluids.

1.2.2 Glutathione metabolism

The interaction between γGT and glutathione in the γ-glutamyl cycle has implied a possible role for the enzyme in regulating tissue glutathione levels. After studying the enzyme from human liver, Shaw and Newman (1979) suggested that γGT played a key role in glutathione catabolism.

1.2.3 Immunoglobulin secretion

γGT preparations obtained from mucous secretions and colostral milk have been shown to contain secretory IgA (Binkley et al., 1975a, 1975b; Binkley and Wiesmann, 1975). These authors suggested that γGT may be the secretory component of IgA. This hypothesis is supported by the fact that high activities of γGT have been demonstrated in cells that are rich in secretory IgA (Albert

et al., 1964; Ross et al., 1973). The evidence, however, is inconclusive and it may be that the function of IgA and γGT, which are distinct from one another, both happen to be associated with secretion and absorption from common surfaces.

1.3 DISTRIBUTION OF γ-GLUTAMYLTRANSFERASE IN TISSUES

γGT has been shown to be present in many of the body tissues and there is general agreement that the highest activity is in the kidney, followed by the pancreas, liver and spleen (Goldbarg et al., 1960; Szczeklik et al., 1961; Orlowski and Meister, 1970). In most other tissues the activity of the enzyme is of the same order of magnitude as that in the plasma that supplies them (Rosalki, 1975).

The earliest investigation into the intracellular location of γGT appears to have been made by Szewczuk (1966). Using high speed centrifugation he fractionated γGT obtained from human liver and kidney into soluble and microsomal fractions comprising 10 and 90% respectively of total tissue activity. The membrane-bound microsomal fraction can be solubilised by the use of detergents such as Triton X-100 and deoxycholate or by treatment with papain (Hughey and Curthoys, 1976).

The enzyme has been purified and extensively characterised from various tissues, in particular liver and kidney (Miller et al., 1976; Hughey and Curthoys, 1976; Huseby, 1977; Tate and Ross, 1977; Masuiki et al., 1982). In all of these studies it was shown to consist of a heavy subunit, relative molecular mass (M_r) 40 000 - 60 000 and a light subunit, M_r 20 000 - 25 000. The papain-solubilised γGT from rat kidney is soluble in aqueous solutions whereas the Triton-solubilised is soluble only in the presence of detergents (Hughey and Curthoys, 1976; Hughey et al., 1979). Papain treatment of the Triton-solubilised enzyme produces a form identical to the papain-solubilised enzyme. The light subunits of the papain and Triton-solubilised enzymes each have an identical M_r whereas the heavy subunit of the latter is about 52 amino acid residues larger than the heavy subunit of the former. From these findings it has been suggested that the papain-sensitive amino terminal sequence of

amino acids of the large subunit is responsible for the membrane-association of γGT (Tate and Meister, 1981).

1.4 γ-GLUTAMYLTRANSFERASE IN FLUIDS

1.4.1 Seminal fluid

Very high levels of γGT are present in seminal fluid and the activity is thought to arise more from spermatozoa than from prostate (Rosalki and Rowe, 1973).

1.4.2 Urine

Urine contains γGT with activities being 2 - 6 times the levels seen in plasma. It is thought not to originate from plasma but from the normal aging of the renal tract cells (Rosalki, 1975).

1.4.3 Bile

High values of γGT activity have also been reported to be present in hepatic bile (Szczeklik et al., 1961; Orlowski, 1963; Rutenburg et al., 1963; Wenham, 1976). It is thought to originate from the cells of the liver by a process of wear and tear.

1.5 SERUM γ-GLUTAMYLTRANSFERASE IN HEALTH AND DISEASE

It is now generally believed that γGT was the agent responsible for the breakdown of glutathione by serum described by Woodward (1939). γGT activity per se was first demonstrated in 1960 by two independent groups of workers (Goldbarg et al., 1960; Szewczuk and Orlowski, 1960). Most studies have shown that serum γGT activity is higher in males than in females (Goldberg, 1980).

Serum γGT activity is raised in a large number of diseases. However, despite the relative activity of γGT in kidney being over 25 times the activity in liver (Orlowski and Meister, 1970), elevations of serum γGT activity are usually associated with liver disease and not kidney disease (Lum and Gambino, 1972).

Serum γGT activity is often increased in patients receiving enzyme-inducing drugs in circumstances where liver damage is unlikely (Ewen and Griffiths, 1973; Bartels et al., 1975a; Skillen and Pierides, 1976). The increase is believed to be due to microsomal enzyme induction and a good correlation has been shown between the urinary excretion of D-glucaric acid (an established index of microsomal enzyme induction) and serum γGT activity in this type of patient (Bartels et al., 1975a). Other drugs are also thought to induce γGT activity. Whitfield et al. (1973) noted an elevated serum γGT activity in a patient receiving warfarin, a recognised enzyme inducing drug (Goldberg, 1980). Similarly, Bartels et al. (1975b), demonstrated that serum γGT activities were elevated in children receiving the anti-inflammatory drug aminopyrine.

The largest increases in serum γGT activity are observed in obstructive liver disease, with values averaging 12 times the upper limit of the reference range (Szczeklik et al., 1961; Rutenburg et al., 1963). In an extensive study of patients with varying degrees of biliary obstruction, Lukasik and Richterich (1965) found that the observed elevations of serum γGT and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, ALP) correlated with one another to a significant extent. Similar findings were reported by Whitfield et al. (1972). Serum levels of γGT activity correlate to some extent with the degree of cholestasis, being higher in complete than in partial obstruction (Rutenburg et al., 1963; Kokot and Kuska, 1968).

Serum γGT activity is also often raised in patients with primary and metastatic carcinoma of the liver (Szczeklik et al., 1961; Rutenburg et al., 1963; Boone et al., 1974) and may be the only abnormal finding in some patients with these conditions (Goldberg, 1980). Several authors have reported that serum γGT levels show a similar behaviour to those of 5 ' nucleotidase (5'-nucleotide phospholydrolase, EC 3.1.3.5, 5'NT) in patients with metastatic liver disease. Korstein et al. (1974), after studying serum levels of both

enzymes sequentially, reported that they paralleled one another. Kolaric et al. (1975) in a series of 30 patients with histologically proven metastatic liver disease showed serum γGT and 5'NT activities to be elevated in all 30 patients whereas positive liver scans were evident in only 20 out of the 30. In another study of 79 patients, Chaimoff et al. (1975) showed an increased serum γGT activity in all but 2 of the patients where liver metastases were proven. In contrast, some workers (Baden et al., 1971; Kim et al., 1977) have concluded that measurement of serum γGT activity was too non-specific in the diagnosis of liver secondaries, with too many false positives. It has been suggested (Goldberg, 1980) that the measurement of serum γGT activity may be of greater value in excluding liver secondaries rather than detecting their presence.

Serum γGT activity is elevated in diseases associated with liver cell damage, as well as those associated with partial or complete biliary obstruction. Villa et al. (1966) and Keane et al. (1973) showed that almost all patients with viral hepatitis had a raised serum γGT activity. However, the degree of elevation did not correlate with that of ALT or asparate aminotransferase (EC 2.6.1.1, AST) (Whitfield et al., 1972). The measurement of γGT may be of prognostic value since it has been suggested that if γGT activity fails to return to normal after viral hepatitis, then the patient is more likely to develop cirrhosis or chronic active hepatitis (Villa et al., 1966; Ideo and Dioguardi, 1970; Jacobs, 1972).

Perhaps the clinical value of serum γGT is greatest in screening patients for alcoholic liver disease or in monitoring whether they abstain from alcohol (Rosalki et al., 1970; Rosalki and Rau, 1972; Rollason et al., 1972). In a study of 146 patients, Whitehead et al. (1978) found that serum γGT activities reflected alcohol consumption. In general, γGT levels return to normal when drinking ceases as long as there is no histological evidence of liver damage (Rosalki et al., 1970; Rosalki and Rau, 1972; Wu et al., 1976).

Serum γGT activities may also be raised in non-hepatic disorders. Patients with extensive burns tend to show greater increases in serum γGT than those with small burns (Coombes et al., 1978). The authors suggested that the increase was due to increased protein catabolism associated with renal failure.

After myocardial infarction, serum γGT activity rises slowly, compared with the activities of other enzymes, to reach a peak at about the 10th day after the onset of the episode (Agostini et al., 1965; Hedworth-Whitty et al., 1967; Ravens et al., 1969; Rosalki et al., 1970; Szczeklik et al., 1972). The mechanism is not known with certainty but Agostini et al. (1965) suggested, and this has been supported by others (Ewen and Griffiths, 1971; Szczeklik et al., 1972) that it is due to an increased rate of γGT synthesis. This occurs due to the repair of the myocardium and, in particular, the vascular endothelium which is rich in γGT (Ravens et al., 1969). However, others have suggested it to be due to liver damage secondary to congestive cardiac failure (Betro et al., 1973; Cooke and Carter, 1973; Connell, 1973).

In summary, serum γGT activity is elevated in all forms of liver disease, but its measurement is of little discriminatory value. However, γGT is probably the most sensitive overall index of liver disease presently available.

1.6 THE CONCEPT OF ISOENZYMES AND MULTIPLE ENZYME FORMS

It has for a long time been known that certain enzymes occur in different forms in different tissues; for example, prostatic acid phosphatase may be distinguished from the erythrocytic enzyme by its marked inhibition by L-(+)-tartrate. From the middle 1950's onwards there was considerable improvement of separative techniques using net molecular charge. Interest was particularly stimulated by the application of zone electrophoresis using starch and later polyacrylamide gel as the supporting medium together with the adaptation of histochemical methods to visualise the separated enzyme forms within the gel (Hunter and Markert, 1957). In 1959, Markert and Moller introduced the term 'isozyme' to denote the different molecular forms in which proteins with similar enzyme specificity may exist. Some authors preferred the term 'isoenzyme' and consequently the 2 terms have been used interchangeably. The Commission on Biological Nomenclature of the International Union of Biochemistry Commission on Biochemical Nomenclature (1977) has approved both terms but has recommended that their use be restricted to those enzymes whose occurrence as multiple forms arises from genetically determined differences in the primary structure, due to the

presence of more than one structural gene. Those enzymes variants that arise by post-transcriptional modification, due to association with variable amounts of charged moieties or polymerisation, are not considered to be isoenzymes and the term 'multiple forms' is considered appropriate. The Commission recommended labelling these multiple forms on the basis of their electrophoretic mobility. For these reasons the multiple forms of γGT described and studied further in this thesis will be referred to as multiple forms. At present there is no evidence to suggest that they arise from different structural genes and so the term isoenzyme is considered inappropriate.

1.7 MULTIPLE FORMS OF γ-GLUTAMYLTRANSFERASE IN HEALTH AND DISEASE

1.7.1 Electrophoresis in different media

Many different electrophoretic media have been used to separate the multiple forms of γGT present in serum. Broadly speaking, they are of two types; (a) those that separate mainly on the basis of charge differences; (b) those which combine charge differences with molecular sieving effects. Paper, cellulose acetate, agar and agarose come into the first category and starch gel and polyacrylamide gel are included in the second.

Serum from healthy individuals: Using electrophoresis on paper, Kokot and Kuska (1965) first demonstrated that in healthy subjects most of the serum γGT activity is present as two bands, one in the α_1- and the other in the α_2-globulin region. Subsequent studies, using a variety of media have reported similar findings (Jacyszyn and Laursen, 1968; Miyazaki and Okumura, 1972; Patel and O'Gorman, 1973; Azzopardi and Jayle, 1973; Hetland et al., 1975; Kok et al., 1978; Burlina, 1978; Wenham et al., 1979).

Serum from patients with liver disease: In contrast to the situation in healthy individuals, much confusion exists as to the number of bands present in the serum of patients with liver disease. The confusion is made worse because few

studies have attempted to relate the bands obtained using different media to one another. The only common findings appear to be that; (a) the two bands present in normal sera are also present in liver disease but usually in increased amounts; (b) bands of additional mobility are also present (Kokot and Kuska, 1965; Orlowski and Szczeklik, 1967; Kokot and Kuska, 1968; Rutenburg et al., 1967; Rosalki et al., 1970; Lehmann et al., 1970; Miyazaki and Okumura, 1972; Patel and O'Gorman, 1973; Hetland et al., 1975; Degenaar et al., 1976; Kok et al., 1978; Burlina , 1978; Wenham et al., 1979).

Bile: In comparison to serum, few electrophoretic studies have been carried out on human bile and the literature is somewhat less confusing. Using agarose, Jacyszyn and Laursen (1968) demonstrated activity in the β-globulin position following electrophoresis of hepatic bile obtained from 'T' tube drainage. Wenham et al. (1978a) also detected a band in this position, as well as two additional bands in the albumin-α_1- and α_2-globulin regions. Polyacrylamide gel electrophoresis of hepatic bile results in most γGT activity remaining at the origin together with a minor zone with mobility between 70 and 80% of that of albumin (Huseby, 1978; Wenham et al., 1978a).

1.7.2 Gel filtration chromatography

Serum: Gel chromatography of serum with normal γGT activity on Sephadex G200 (Orlowski et al., 1965; Orlowski and Szczeklik, 1967) shows three peaks of activity of high, intermediate and low M_r (greater then 600 000, 250 000 - 450 000 and about 100 000 respectively). In liver disease, most workers also find three peaks of high, intermediate and low M_r (Orlowski et al., 1965; Kokot and Kuska, 1968; Wenham et al., 1979). However, Ideo and co-workers (Ideo and Ronchi, 1972; Ideo et al., 1972) found only one peak, which eluted in the void volume on Sephadex G200 and whose M_r they estimated to be 200 000. Their findings seem difficult to interpret since proteins that elute in the void volume of Sephadex G200 have an M_r of at least 600 000. In obstructive liver disease the elevation in serum γGT activity appears to be associated mainly with an increase in the peak of high M_r (high M_r γGT) (Orlowski et al., 1965; Orlowski and Szczeklik, 1967; Kokot and Kuska, 1968; Wenham et al., 1979). However, no clinical evaluations appear to have been made of the measurement of this fraction as an aid in the differential diagnosis of liver disease.

In patients with viral hepatitis the increase in serum γGT activity was found to be evenly distributed between the high and intermediate M_r peaks (Orlowski et al., 1965) although Kokot and Kuska (1968) found it to be associated only with the high M_r γGT peak.

Bile: Fewer studies have been made on hepatic bile but all confirm that the vast majority of the enzyme is present as high M_r γGT (Indirani and Hill, 1977; Huseby, 1978; Wenham et al., 1978a).

1.7.3 Relationship between the fractions obtained by electrophoresis and gel chromatography

Serum: In contrast to the large number of studies using one or other technique, few attempts have been made to relate the fractions obtained by gel chromatography to the bands seen on electrophoresis. Of the few studies made, the main conclusion appears to be, (1) that the high M_r peak remains at the origin after polyacrylamide or starch gel electrophoresis, (2) the intermediate M_r peak travels in the α_1-globulin region, and (3) the low M_r peak travels in the α_2-globulin region (Orlowski and Szczeklik, 1967; Wenham et al., 1979).

1.8 WHY DOES SERUM γGT ACTIVITY RISE IN LIVER DISEASE?

Enzymes such as ALT, that are present in the soluble cytoplasm of the liver cell, are believed to be released into the circulation by simple leakage of the cell contents following hepatocellular injury. In contrast, γGT is largely membrane-bound and so this simple explanation is not applicable. Several alternative theories have been proposed to explain the release of γGT into the circulation in liver disease. For clarity they have been categorised in terms of the physical processes that are thought to occur:

(1) The retention theory (Rutenburg et al., 1963). This proposed that the increase in serum γGT activity in liver and biliary-tract disease was due to the failure of the normal excretion of the enzyme in the

bile. However, the earlier suggestions that biliary excretion was a factor in the clearance of plasma enzymes such as ALP have not been supported by recent work. For example, injection of purified human placental ALP (measured by utilising its heat stability) into normal subjects and patients with biliary obstruction resulted in decay curves in serum that did not differ from those of other plasma proteins and which were similar in the two groups (Clubb et al., 1965). It is, therefore, unlikely that accumulation of γGT from either hepatic or non-hepatic sources occurs due to biliary stasis, and so to a large extent the retention theory has been abandoned.

(2) The hepatogenic theory (Orlowski, 1963). This proposes that hepatic γGT synthesis (and subsequent release) can be induced by certain liver diseases.

(3) The membrane fragment theory (Shinkai and Akedo, 1972; De Broe et al., 1975). This suggests that in liver disease, fragments of liver cell membranes to which γGT, ALP, adenosine triphosphatase (EC 3.6.1.4, ATPase) and other proteins are attached, may break off the main membrane. These particulate fragments may then, by a process which remains rather obscure, find their way into the circulation.

(4) The theory of elution (Moss, 1980). This proposes that if γGT is an enzyme of the outer surface of cellular membranes, it may be eluted from the cellular surface into the plasma in certain disease states. At present however, the mechanism behind such an elution process remains unknown.

None of these 4 theories, however, has yet gained widespread acceptance and so the mechanisms behind the rise in serum γGT activity in liver disease are unknown.

1.9 OTHER ENZYMES SHOWING INCREASED SERUM ACTIVITY IN LIVER DISEASE

Leucine aminopeptidase (aminopeptidase (microsomal), EC 3.4.11.2, LAP) and ALP are two other enzymes, whose serum activities are elevated in liver disease. Like γGT they not only show particularly high levels of activity in obstructive liver disease but also originate from the hepatocyte plasma membrane.

1.9.1 Leucine aminopeptidase

Leucine aminopeptidase catalyses the reaction:

$$\text{RNH- leucine} + H_2O \rightleftharpoons \text{leucine} + RNH_2$$

R may be a peptide or an amino acid and the leucine moiety may be replaced by other amino acids although the enzyme is not as efficient at hydrolysing these. Although the recommended name for the enzyme is aminopeptidase (Enzyme Commission recommendations, 1972), leucine derivatives are the commoner substrates in its determination and leucine aminopeptidase is the term used most frequently in the literature. For these reasons the term leucine aminopeptidase is used throughout this thesis.

Leucine aminopeptidase is present in most human tissues, particularly the cells of the liver and biliary tract (Nachlas et al., 1957; Kowlessar et al., 1960) and it is located mainly on the plasma membrane (Emmelot et al., 1968).

Like γGT, LAP is present in serum. Serum activities are raised in a wide variety of liver diseases (Harkness et al., 1960; Pineda et al., 1960; Kowlessar et al., 1961; Batsakis et al., 1968). The highest values occur in extrahepatic biliary obstruction (Horky et al., 1967, Batsakis et al., 1968).

Leucine aminopeptidase occurs in multiple forms in serum and bile but it is not known whether these represent true isoenzymes (see section 1.6).

Electrophoresis of normal serum on paper or cellulose acetate gives rise to a single band of LAP activity in the α_1-globulin region (Smith et al., 1962; Meade and Rosalki, 1964). Extra bands appear in the albumin, α_2- and β-globulin region in liver disease.

In a study of patients receiving anticonvulsant drug therapy, Rundle and Sudell (1973) obtained up to 5 types of LAP pattern after performing serum electrophoresis on cellulose acetate. They concluded that LAP electrophoresis was of little clinical value. After electrophoresis on starch gel, large amounts of LAP activity are obtained at the origin in patients with liver disease, and this is especially so in patients with obstructive jaundice (Kowlessar et al., 1960; Kowlessar et al., 1961).

Electrophoresis of hepatic bile gives rise to a single LAP band, coincident with the band observed in normal serum (Kowlessar et al., 1960; Kowlessar et al., 1961).

After gel chromatography on Sephadex G200 2 peaks of LAP activity are obtained, eluting with the 19S and 7S protein peaks (Ideo and Ronchi, 1972). The 19S peak appears to contribute a greater proportion towards total activity in lesions involving cholestasis. LAP activity is also obtained in the void volume after gel chromatography of sera from patients with hepatic cancer on Sepharose 4B (Shinkai and Akedo, 1972).

1.9.2 Alkaline phosphatase

Alkaline phosphatase catalyses the reaction:

$$ROHPO_3H_2 + H_2O \rightleftharpoons ROH + HOPO_3H_2$$

R may be any aliphatic, aromatic or heterocyclic grouping or even an artificial chromogen.

Alkaline phosphatase is present in many human tissues with large amounts being found in liver, intestine, bone, spleen and placenta. Normal serum also

contains ALP activity, and this is raised in liver disease, especially extrahepatic biliary obstruction and liver metastases, and in bone disease, e.g. Paget's disease and osteomalacia.

There are three isoenzymes of ALP, as defined in section 1.6, namely those from liver/bone, intestinal and placental sources (McKenna et al., 1979). Multiple forms of the liver band may be present in serum.

Electrophoresis of normal serum usually shows a predominant liver ALP band, a minor bone band and sometimes one of intestinal origin (see below). The liver isoenzyme has the same mobility as the α_2-globulins following electrophoresis of normal serum on paper, cellulose acetate or agar (Baker and Pellagrino, 1954; Wieme and Demeulanaere, 1970; Price and Sammons, 1974). After electrophoresis on starch or polyacrylamide gel it forms a narrow band with about half of the mobility of albumin (Moss et al., 1961; Hodson et al., 1962; Hill and Sammons, 1967; Price and Sammons, 1974).

In patients with liver disease, the liver band is increased in intensity and another fraction is usually present. This fraction travels ahead of the normal liver band on cellulose acetate and agar, and remains at the origin on polyacrylamide and starch gel (Kowlessar et al., 1959; Hill and Sammons, 1967; Wieme and Demeulenaere, 1970; Price and Sammons, 1974).

The bone band may usually be seen as a diffuse zone running immediately behind the liver band on the different media. In healthy individuals and in patients with liver disease it is usually present as a minor band but is usually the main fraction present in patients with bone disease. When present, the intestinal band runs well behind the liver and bone bands on the different media.

Electrophoresis of hepatic bile shows a main ALP band remaining at the origin on polyacrylamide gel but travelling ahead of the liver band, in the α_1-globulin region on cellulose acetate (Price and Sammons, 1974; Fritch and Adams-Park, 1972).

After gel chromatography of normal serum a main peak of ALP activity is observed eluting with the 7S proteins with an $\underline{M}_r$ estimated to be 200 000 (Estborn, 1964; Price and Sammons, 1974). In patients with liver disease this fraction is increased, along with a fraction of high $\underline{M}_r$ and which is present in large amounts, that elutes in the void volume on Sepharose 4B or Sephadex G200 (Fennelly et al., 1969; Shinkai and Akedo, 1972; Price and Sammons, 1974; Fritch and Adams-Park, 1974). In hepatic bile nearly all of the ALP activity is of high $\underline{M}_r$, eluting in the void volume after gel chromatography on Sephadex G200 (Price and Sammons, 1974).

1.10 AIMS OF THE STUDY

In broad terms, the aims of the study were two-fold. Firstly, an investigation of the physical properties of γGT in bile and serum and secondly, a clinical assessment of the measurement of the multiple forms of γGT in patients with liver disease. The other hepatocyte plasma membrane enzymes LAP and ALP were included in the study, where it was considered appropriate, to see whether the findings for γGT were applicable on a wider basis.

The multiple forms of γGT present in serum might a) represent true isoenzymes, b) consist of aggregates of low $\underline{M}_r$ γGT with lipid and protein (Huseby, 1978) or c) consist of membrane fragments (De Broe et al., 1975). Experiments were designed involving electrophoretic, chromatographic, immunochemical and other physico-chemical techniques to investigate the physical properties of γGT in serum and in bile. It was hoped that the results obtained might provide evidence for or against the above theories.

The details of the study were as follows:

1. To investigate the nature of γGT in hepatic bile.

2. To investigate the nature of serum γGT in health and disease.

3. To undertake a clinical evaluation of the multiple forms of serum γGT in liver disease.

4. To compare the physical properties of γGT in human liver, with the enzyme in bile and serum and to undertake preliminary in vitro elution studies in an attempt to test the elution theory.

The results of the above investigations each form a complete chapter of this thesis. At the end of each of these chapters I have discussed my findings in relation to the results of similar investigations carried out by other workers.

In the final chapter under the heading 'General Discussion' I have attempted to tie the results of each of the chapters together on a more general basis, and relate them to current theories for the existence of γGT in bile and serum.

Chapter 2

ANALYTICAL METHODS

This chapter describes the general methods used throughout the work presented in this thesis. The validation and verification of methods is included, where appropriate, in this section to ease the flow of the text in subsequent sections. Where a method is used only once, for a particular experiment, it is described at the appropriate point in the text.

2.1 MATERIALS

2.1.1 Measurement of enzyme activities

γ-Glutamyltransferase: γ-Glutamyl-p-nitroanilide from Boehringer, Lewes, Sussex, U.K. (Boehringer). Tris (hydroxymethyl) methylamine (Analar grade) from British Drug Houses Chemicals Ltd., Poole, Dorset, U.K. (B.D.H.). Glycylglycine from Koch Light, Colnbrook, Bucks, U.K. (Koch Light).

Leucine aminopeptidase: L-Leucine-p-nitroanilide from Sigma Chemical Co., Poole, Dorset, U.K. (Sigma).

Alkaline phosphatase: p-Nitrophenyl phosphate from Boehringer. 2-Amino-2-methyl-propan-1-ol, diethanolamine and magnesium chloride from B.D.H..

2.1.2 Fractionation of multimolecular enzyme forms

Electrophoretic media: Acrylamide, N:N'-methylene-bis-acrylamide from Eastman Kodak, Liverpool, U.K. (Kodak). 4-30% Polyacrylamide gradient gels from Pharmacia, Uppsala, Sweden (Pharmacia).

Enzyme staining reagents: γ-L-Glutamyl-α-naphthylamide, L-leucyl-β-naphthylamide-HCl from Koch Light. α-Naphthyl acid phosphate (potassium salt), Fast Blue B and 4-aminodiphenylamine diazonium sulphate from Sigma. Glacial acetic acid (Analar grade) from B.D.H..

Gel Chromatography: Sephadex G200 and Sephacryl S300 from Pharmacia.

2.1.3 Physical and biochemical properties

Thyroglobulin, ferritin, catalase, aldolase and lactate dehydrogenase from Pharmacia. Papain (twice crystallised), sodium deoxycholate, sodium glycocholate, sodium glycochenodeoxycholate, 2,5-diphenyl oxazole and 1,4 -di (2-(5-phenyl oxazoyl)-benzene from Sigma. Triton X-100 from B.D.H.. Bacto-agar from Difco laboratories, Detroit, Michigan, U.S.A.. Agarose E.F. from LKB, Croydon, U.K.. Antisera to apolipoprotein A and apolipoprotein B from Hoechst, Hounslow, Middlesex, U.K.. Naphthalene black from George Gurr Ltd., London. Fat Red B from Corning ACI, Palo Alto, California, U.S.A. (Corning). Dextran sulphate ($\underline{M}_r$ = 15 000) from Sochibo, Boulogne, France. All other chemicals were of analytical grade from B.D.H..

2.1.4 Equipment

8600 Reaction rate analyser, chromatography columns, Uvicord II absorptiometer and recorder, 2070 Ultrorac II fraction collector, 1200 Varioperpex peristaltic pump and 2103 electrophoresis power supply from LKB, Bromma, Sweden. Sequential Multiple Analyser plus Computer (S.M.A.C.) from Technicon, Basingstoke, U.K.. Electrophoresis tank for polyacrylamide vertical gel slabs from Raven Scientific, Haverhill, Suffolk, U.K.. Electrophoresis tank for polyacrylamide gradient gels from Universal Scientific Ltd., London, U.K.. SP800 spectrophotometer from Pye-Unicam, Cambridge, UK.. Prepspeed 65 ultracentrifuge from M.S.E., Fisons Scientific, Loughborough, Leicestershire, U.K.. Sorvall RC 2B superspeed centrifuge from Dupont Instruments, Newtown, Connecticut, U.S.A..

2.2 MEASUREMENT OF ENZYME ACTIVITIES

2.2.1 γ-Glutamyltransferase

Principle of method: γGT catalyses the following reaction:

γ-L-glutamyl-p-nitroanilide + glycylglycine ⇌ p-nitroaniline + γ-L-glutamyl-glycylglycine

This was measured in serum and bile using the method of Rosalki and Tarlow (1974).

50 µl of serum or bile was mixed with 1.0 ml of Tris (115 mmol/l) - glycylglycine (138 mmol/l) buffer, pH 8.5. The reaction was initiated by the addition of 100 µl of γ-L-glutamyl-p-nitroanilide (104 mmol/l) dissolved in 0.5 mol/l HCl. The final reaction conditions were:

γ-L-glutamyl-p-nitroanilide	9.0 mmol/l
Tris	100 mmol/l
glycylglycine	120 mmol/l
pH	8.0

The formation of p-nitroaniline was monitored at 410 nm using an LKB reaction rate analyser. A molar extinction coefficient of 8500 for p-nitroanaline was used in the calculations (Wenham, 1976).

The method was modified for the measurement of γGT activity in column fractions. 0.5 ml of column effluent was mixed with 0.5 ml of Tris-glycylglycine buffer pH 8.5, the composition of which had been adjusted to give the same final reaction conditions as set out above.

Within-batch precision was determined by making 20 replicate measurements of enzyme activity both in a commercial quality control material and also a pool of column effluent. Between-batch precision was determined from 20

measurements of enzyme activity in the same materials as above, each measurement being made on a different day. The precision figures for the assay are shown in Table 2.1.

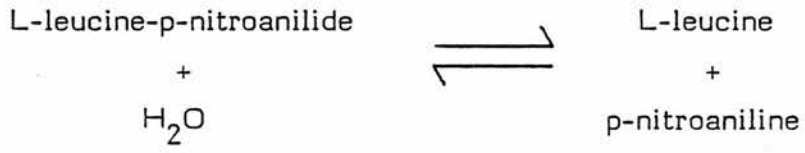
Table 2.1

Precision of the various enzyme estimations

Enzyme		Within-batch		Between-batch	
	mean (U/l)	S.D. (U/l)	C.V. (%)	S.D. (U/l)	C.V. (%)
γGT (Serum, bile)	135	7.0	5.2	7.3	5.4
LAP (serum, bile)	185	7.2	3.9	9.1	4.9
ALP (serum)	177	3.4	1.9	3.5	2.0
ALP (bile)	112	2.7	2.4	3.4	3.0
γGT (column fractions)	7.6	0.19	2.5	0.3	4.0
LAP (column fractions)	3.8	0.22	5.7	0.3	7.9
ALP (column fractions)	1.3	0.078	6.0	0.085	6.5

2.2.2 Leucine aminopeptidase

Principle of method: LAP catalyses the reaction:



Leucine aminopeptidase activity was measured in serum and bile using a modification of the method of Szasz (1967).

50 µl of sample was mixed with 1.0 ml of Tris (100 mmol/l)-HCl buffer, pH 7.7. The reaction was initiated by the addition of 50 µl of L-leucine-p-nitroanilide (33.6 mmol/l), dissolved in 0.25 mol/l HCl. The final reaction conditions were:

L-leucine-p-nitroanilide	1.5 mmol/l
Tris	90.9 mmol/l
pH	7.5

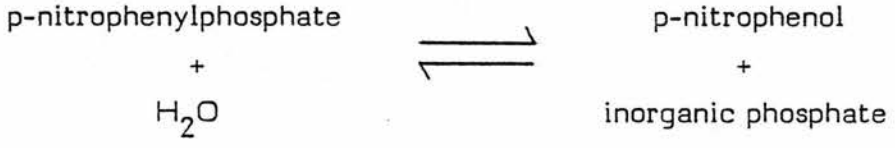
The liberated p-nitroaniline was monitored at 410 nm in an LKB 8600 reaction rate analyser. A molar extinction coefficient of 8500 for p-nitroaniline was used in the calculations (Wenham, 1976).

The method was modified to measure enzyme activity in column fractions. 0.5 ml of effluent was mixed with 0.5 ml of Tris-HCl buffer, pH 7.7, the composition of which had been altered so as to give the same final reaction conditions as set out above.

The precision figures for the measurement of LAP activity in serum, bile and column fractions are shown in Table 2.1.

2.2.3 Alkaline Phosphatase

Principle of method: The reaction catalysed by alkaline phosphatase is:



This was measured in serum using a S.M.A.C.. The substrate was 10 mmol/l p-nitrophenyl phosphate in 0.6 mol/l 2-amino-2-methyl-1-propanol buffer, pH 10.2, containing 0.5 mmol/l magnesium chloride (Bowers and McComb, 1966).

Alkaline phosphatase activity was measured in hepatic bile using the method of McComb and Bowers (1972).

50 µl of bile was mixed with 1.0 ml of diethanolamine buffer (0.9 mol/l, pH 10.2) containing 0.5 mmol/l magnesium chloride. The reaction was initiated by

the addition of 100 µl of p-nitrophenylphosphate (154 mmol/l) dissolved in 0.9 mol/l diethanolamine, pH 10.2, containing 0.5 mmol/l magnesium chloride. The final reaction conditions were:

p-nitrophenylphosphate	12.8 mmol/l
diethanolamine	0.9 mol/l
magnesium chloride	0.5 mmol/l
pH	10.2

The liberation of p-nitrophenol was monitored at 410 nm in an LKB 8600 reaction rate analyser. A molar extinction coefficient of 18,800 for p-nitrophenol was used in the calculation (McComb and Bowers, 1972).

Enzyme activity in column fractions was measured after mixing 0.5 ml of effluent with 0.5 ml of diethanolamine buffer (1.8 mol/l), pH 10.2, containing 1.0 mmol/l magnesium chloride; the final concentrations being the same as set out above.

The precision figures for the 3 methods used to measure alkaline phosphatase are shown in Table 2.1.

2.3 GEL CHROMATOGRAPHY

This was performed using either Sephadex G200 or Sephacryl S300.

2.3.1 Sephadex G200 gel chromatography

Reagents: Sephadex G200 was obtained as dry beads. 20 mmol/l Tris-HCl buffer, pH 8.0, at 20°C was used, containing 50 mmol/l sodium chloride to prevent protein trailing. The buffer was degassed prior to use. A 2.6 x 65 cm LKB chromatography column was used.

Preparation of the gel: The dry Sephadex beads were soaked in an excess of buffer for two days prior to use.

Packing of the column: The column was mounted vertically with the bottom outlet leading to a beaker. A packing extension was connected to the top of the column enabling the whole length of the main column to be packed. The main column was then filled with buffer and any air bubbles removed by drawing the buffer up and down through the bottom outlet with a 60 ml syringe. The column was then tilted slightly, the packing extension filled with the gel slurry and the column allowed to pack under gravity for 6 h.

When packing was complete, the extension was removed, the top put on the column and the bottom outlet connected to an LKB peristaltic pump. Buffer was then pumped upwards through the column at a flow rate of 15.6 ml/h for 24 h before use to enable complete equilibration of the gel with buffer.

Sample application: This was achieved using a syringe connected by a 'T' piece to the input buffer line. 2.6 ml fractions were collected using an LKB 2070 Ultrorac fraction collector and the protein content monitored at 280 nm using an LKB Uvicord II flow-through absorptiometer.

2.3.2 Sephacryl S300 gel chromatography

This was performed in a manner similar to that described for Sephadex G200. However, 2.6 x 65 and 2.6 x 96 cm columns were used, the upward flow rate was 28 ml/h and 3.5 ml fractions were collected.

2.3.3 Calibration of the gel columns:

Each Sephadex and Sephacryl column was calibrated by performing gel chromatography on proteins of known M_r. Calibration curves were prepared by plotting their elution volume (linear scale) versus M_r (logarithmic scale). Whenever any alteration in flow rate occurred, due to suspected infection or admission of air bubbles, the column was immediately dismantled, repacked and recalibrated. Each time a column was repacked, a fresh calibration curve was prepared since different columns, although ostensibly of the same dimensions, possessed slightly different elution characteristics. Minor

between-column variations in the elution volumes of the various peaks were observed, but their estimated $\underline{M}_r$ did not differ by more than 5%. The calibration proteins and their methods of detection are listed in Table 2.2 and typical calibration curves are shown in Figs. 2.1 and 2.2.

Table 2.2

Proteins used to calibrate Sephadex G200 and Sephacryl S300 columns

Protein	$\underline{M}_r$	Method of detection
IgM*	900 000	"In house" kinetic immuno-turbidemetry
Thyroglobulin*	669 000	absorption at 280 nm
Ferritin	440 000	absorption at 280 nm
Catalase	232 000	absorption at 280 nm
Aldolase	158 000	absorption at 280 nm
Lactate dehydrogenase	140 000	Pyruvate/NADH substrates; reaction rate 340 nm (Empfehlungen der deutschen Gesellschaft fur klinische Chemie, 1970, 1972)
Albumin	67 000	absorption at 280 nm

* These proteins were not used to calibrate Sephadex G200 columns.

2.3.4 Investigation into the choice of gel matrix for the chromatography of γGT in human serum

Originally it was intended to perform gel chromatography of γGT in human serum using Sephadex G200. This medium has a relatively small mesh size and excludes all molecules of $\underline{M}_r$ greater than 600 000. However, during the course of this study, a newer gel matrix, Sephacryl S300, became generally available. This is an allyl dextran, cross-linked with N,N'-methylene-bis-

Figure 2.1 Typical calibration curve for a 65 x 26 cm column of Sephadex G200. The void volume (elution volume of blue dextran) was approximately 105 ml.

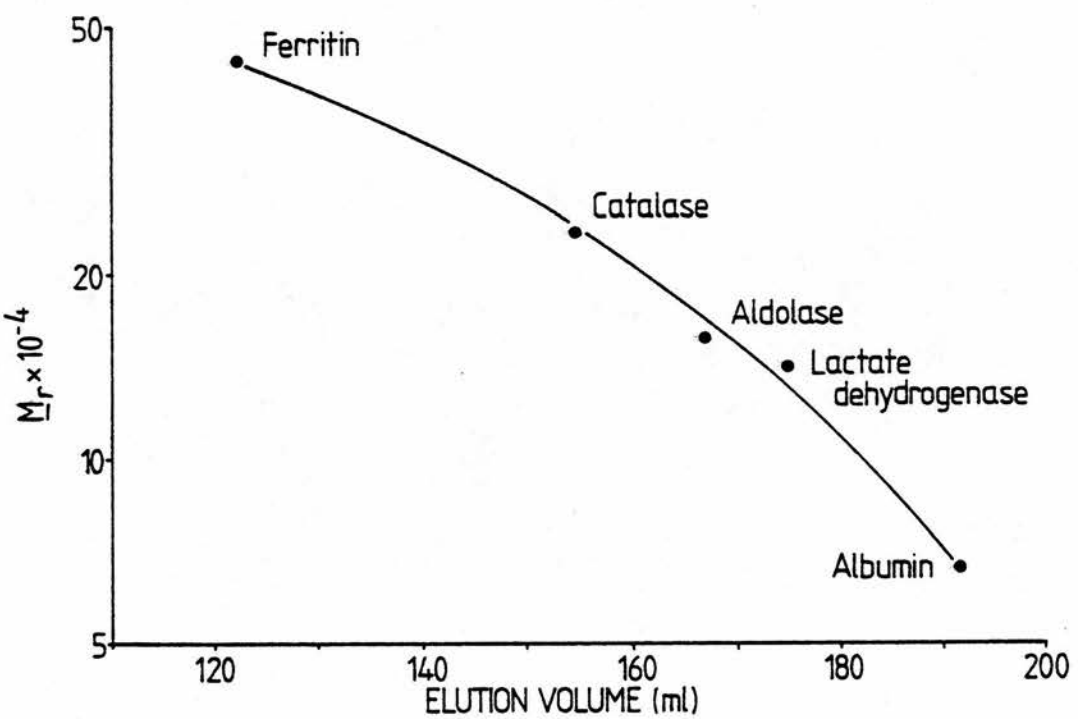
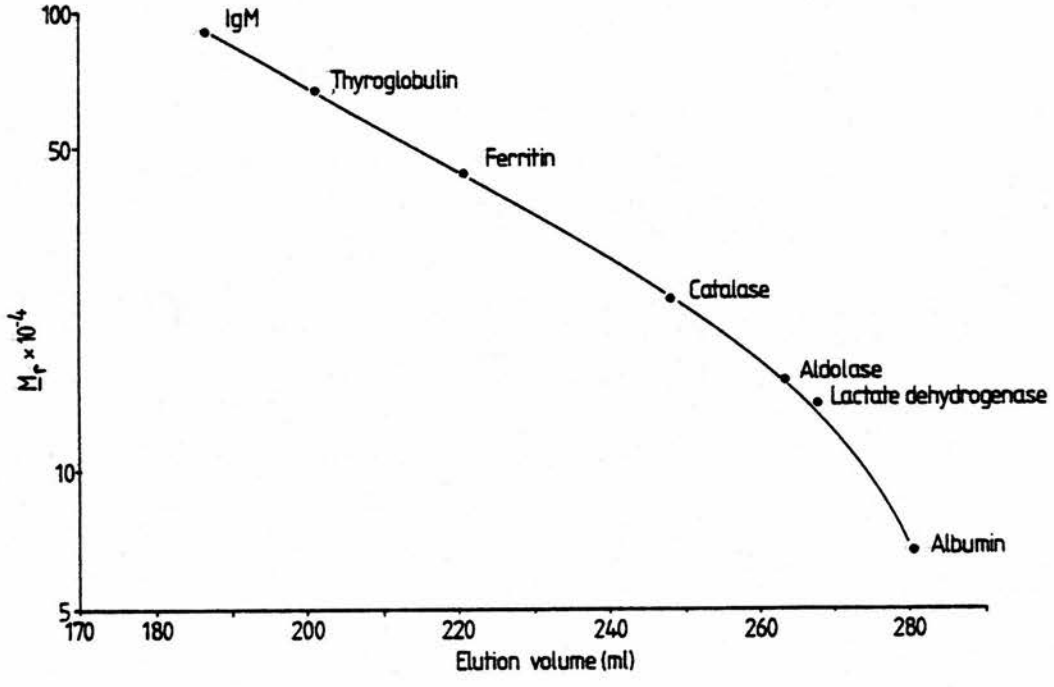


Figure 2.2 Semi-logarithmic plot of elution volume versus M_r in the calibration of a 95 x 2.6 cm column of Sephacryl S300.



acrylamide, giving a rigid gel with a carefully controlled pore size that excludes all molecules of $\underline{M}_r$ greater than 1 000 000. It was decided, therefore, to compare the elution profiles of γGT obtained after performing gel chromatography of human serum on both Sephadex G200 and Sephacryl S300. It was postulated that Sephacryl S300 would give better resolution of those γGT fractions eluting near to or with the void volume than when Sephadex G200 was used.

Procedure: 2 ml of serum from a patient with alcoholic cirrhosis was subjected to both Sephadex G200 and Sephacryl S300 gel chromatography. γGT activity was measured in both eluates and the elution profiles compared.

Results: Chromatography of the serum on 2.6 x 65 cm columns of both media gave rise to three peaks of γGT activity of high, intermediate and low $\underline{M}_r$ (Figs. 2.3 and 2.4). For reasons given in later sections of this thesis, these were called Peak 1 (γGT), Peak 2 (γGT) and Peak 4 (γGT) respectively. Peaks 1 (γGT) and 2 (γGT) were poorly resolved following chromatography on Sephadex G200 but were completely resolved using Sephacryl S300. Although Peaks 2 (γGT) and 4 (γGT) were not as clearly resolved by Sephacryl S300 as by Sephadex G200, it was thought that the resolution could be improved by increasing the column length to 95 cm. Gel chromatography of 1 ml of serum from another patient with alcoholic cirrhosis confirmed this to be the case (Fig. 2.5).

Because Sephacryl S300 gave greater resolution of the γGT fractions, it was chosen as the medium for the chromatography of γGT in human serum.

2.4 ELECTROPHORESIS AND QUALITATIVE LOCALISATION OF MULTIPLE ENZYME FORMS

2.4.1 γ-Glutamyltransferase

Polyacrylamide gel electrophoresis

This was performed in vertical 7% gel slabs (140 x 165 x 3 mm) using the Raven IN96 electrophoresis apparatus and a modification of the method of Azzopardi and Jayle (1973).

Figure 2.3 Elution profile of γGT following gel chromatography on a 65 x 2.6 cm column of Sephadex G200, of serum from a patient with alcoholic cirrhosis.

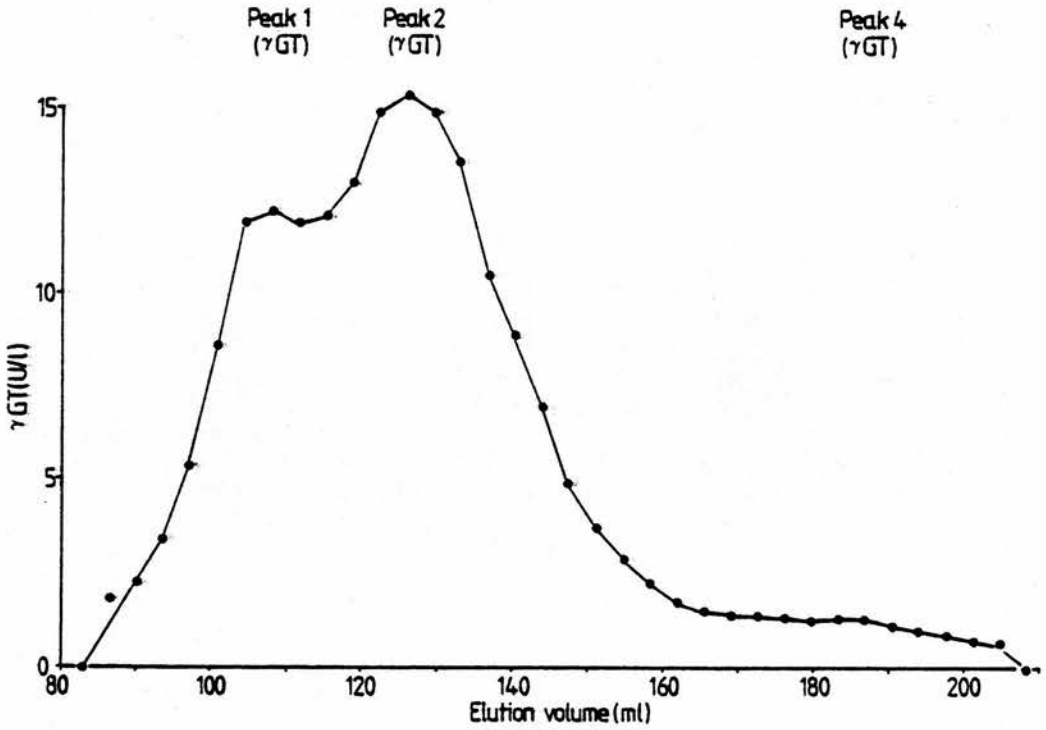


Figure 2.4 Gel chromatography on a 65 x 2.6 cm column of Sephacryl S300 of γGT present in serum from a patient with alcoholic cirrhosis.

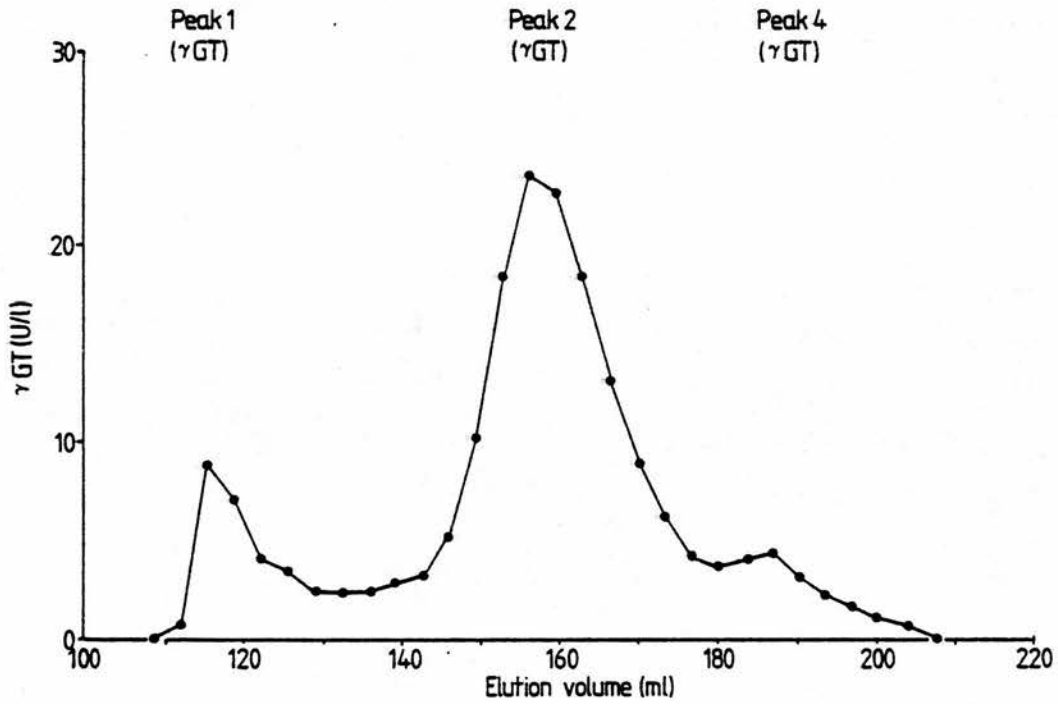
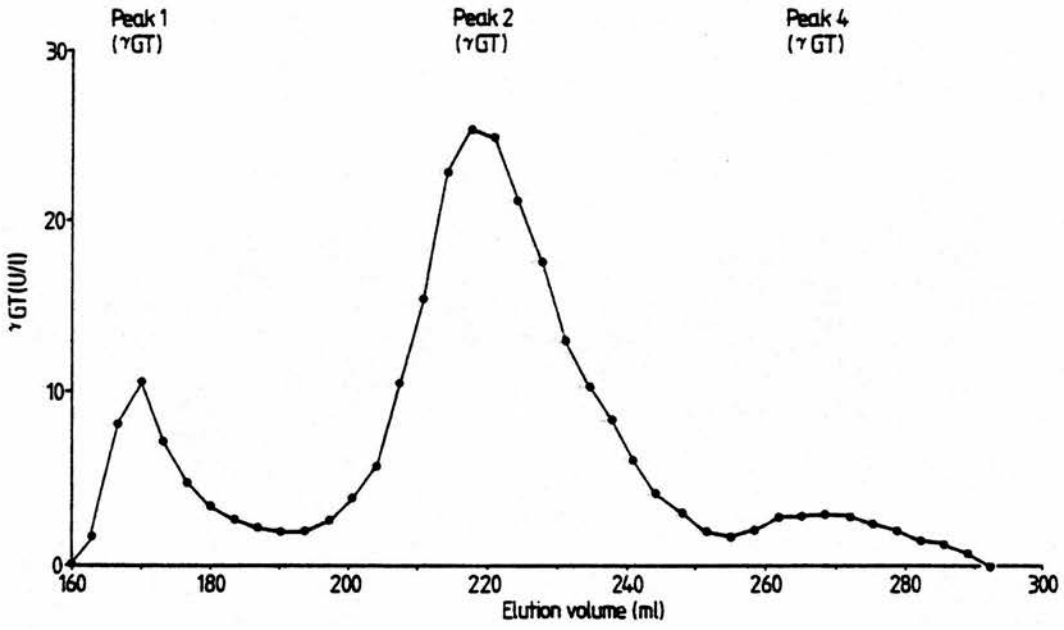


Figure 2.5 Elution profile of γGT following gel chromatography on a 95 x 2.6 cm of Sephacryl S300, of serum from a patient with alcoholic cirrhosis.



Stock gel monomer: 280 g of acrylamide and 7.35 g of N,N'-methylene-bis-acrylamide dissolved in 1 litre of distilled water.

Catalyst: 1.4 g of ammonium persulphate per litre of distilled water.

Gel buffer: Tris (720 mmol/l)-HCl, pH 8.9, containing 2.3 ml of N,N,N',N'-tetramethyl-ethylene diamine (Temed) per litre.

Electrode Buffer (Davis 1964): Tris (50 mmol/l)-glycine (384 mmol/l) pH 8.3, diluted 1 in 10 (v/v) with distilled water immediately prior to use.

Preparation of gel: 1 volume of stock gel monomer, 2 volumes of catalyst solution and 1 volume of gel buffer were mixed and the gel cast. n-Butanol was layered on top of the solution to prevent the inhibition of the polymerisation process by oxygen.

Electrophoresis: This was performed either for 2-3 at a constant voltage of 250 V or overnight at a constant voltage of 70 V.

Polyacrylamide gradient gel electrophoresis

This was carried out on vertical 4 - 30% polyacrylamide gradient gels, pre-equilibrated with Tris (5.0 mmol/l) -glycine (38.4 mmol/l), pH 8.3 for 15 min at 125 V. Electrophoresis was performed for 20 min at 70 V followed by 18 h at 125 V at room temperature.

Localisation of enzyme activity

This was performed in two stages using a modification of the method of Wenham et al. (1978b).

Preparation of substrate-buffer-acceptor solution: 165 mg of γ-L-glutamyl-α-naphthylamide were dissolved in 2 ml of 0.5 mol/l HCl. The solution was mixed with 30 ml of buffer-acceptor solution (Tris (220 mmol/l) - glycylglycine (110 mmol/l), pH 7.4, at 37°C) and the pH brought back to 7.4 by the addition of 1 ml of sodium hydroxide (1 mol/l). The final composition of the solution was:

γ-L-glutamyl-α-naphthylamide	18.5 mmol/l
Tris	200 mmol/l
glycylglycine	100 mmol/l
pH	7.4

Preparation of staining solution: 50 mg of Fast Blue B diazonium salt was dissolved in 50 ml of 10% (v/v) acetic acid.

Procedure: The 7% slab gel or gradient gel was incubated at 37°C for 3 h in a dish containing substrate-buffer-acceptor solution. The gel was washed twice with distilled water and immersed in staining solution for 15 min at room temperature. The gels were placed in distilled water and stored in the dark until photographed.

2.4.2 Leucine aminopeptidase

7% Polyacrylamide gel and polyacrylamide gradient gel electrophoresis

Electrophoresis was carried out exactly as described for γGT.

Localisation of enzyme activity

This was done in 2 stages using a slight modification of the method of Goldbarg and Rutenburg (1958).

Preparation of substrate-buffer solution: 20 mg of L-leucyl-β-naphthylamide were dissolved in 2 ml of 10 mmol/l HCl and the solution added to 50 ml of 200 mmol/l Tris-HCl buffer, pH 7.7. The final compostion of the solution was:

L-leucyl-β-naphthylamide	1.31 mmol/l
Tris	192 mmol/l
pH	7.5

Staining solution: This was prepared exactly as for γGT.

Procedure: This was exactly as for γGT.

2.4.3 Alkaline phosphatase

Polyacrylamide gel electrophoresis

This was performed in uniform vertical 7% gel slabs (140 x 165 x 3 mm) using the Raven IN96 electrophoresis apparatus and a modification (Warwick et al., 1972) of the method of Kaplan and Rogers (1969).

Stock gel monomer: 13.3 g of acrylamide and 0.7 g of N, N'-methylene-bis-acrylamide per litre of distilled water.

Gel and electrode buffer: Tris (370 mmol/l)-borate, pH 9.5, containing 0.5 mmol/l magnesium chloride.

Catalyst and accelerator: 0.07 g of ammonium persulphate and 0.4 ml of Temed per 80 ml of gel-buffer solution.

Casting of gel: Equal volumes (40 ml) of buffer and stock gel monomer were mixed, the appropriate amount of persulphate and Temed added and the gel cast.

Electrophoresis: This was performed either for 2 - 3 h at a constant voltage of 250 V or overnight at a constant voltage of 70 V.

Polyacrylamide gradient gel electrophoresis

4 - 30% polyacrylamide gradient gels, pre-equilibrated with 370 mmol/l Tris-borate buffer, pH 9.5, containing 0.5 mmol/l magnesium chloride, for 15 min at 125 V, were used. Electrophoresis of the samples was performed for 20 min at 70 V, followed by 18 h at 125 V at room temperature.

Localisation of enzyme activity

This was performed in a single stage using α-naphthyl acid phosphate as substrate.

Substrate-staining solution: 39.2 mg of α-naphthyl acid phosphate and 100 mg of 4-amino-diphenylamine diazonium sulphate were dissolved in 100 ml of Tris-borate buffer. The final composition of the solution was:

α-naphthyl acid phosphate	0.75 mmol/l
4-amino diphenylamine diazonium sulphate	2mmol/l
magnesium chloride	0.5 mmol/l
pH	9.5

Procedure: The 7% slab or gradient gel was incubated for 3 h at 37°C in a dish containing the buffer-substrate-staining solution. The gels were then cleared by immersion in 10% (v/v) acetic acid solution.

2.4.4 Calibration of the polyacrylamide gradient gels

Each gradient gel was calibrated by running a mixture of proteins of knowm M_r in one of the slots on the gel. At the end of each run, the track corresponding to the marker proteins was sliced from the rest of the gel and stained for protein by immersion in a solution of 1% naphthalene black in glacial acetic acid-absolute methanol (10/90 (v/v)). The gel was then destained in pure solvent for 24 h and the migration distance of each marker protein measured. A calibration curve was constructed by plotting migration distance against log M_r. The proteins used for calibration are listed in Table 2.3 and a typical calibration curve is shown in Fig. 2.6. To reduce between-gel variation to a minimum, the mixture of calibration proteins was run with every gel.

Figure 2.6 Calibration graph of 4 - 30% polyacrylamide gradient gel.

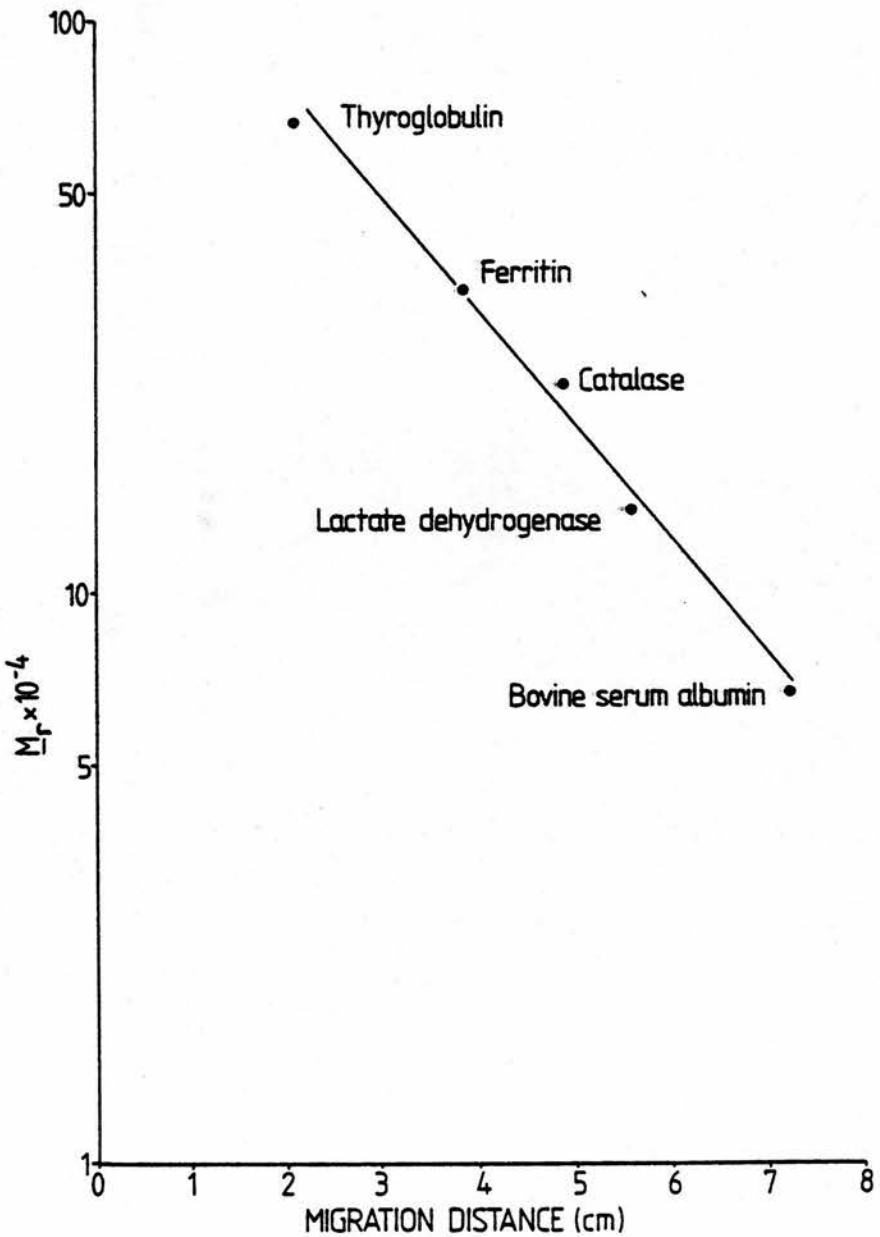


Table 2.3

Proteins used to calibrate polyacrylamide gradient gels

Protein	M_r
Bovine serum albumin	67 000
Lactate dehydrogenase	140 000
Catalase	232 000
Ferritin	444 000
Thyroglobulin	669 000

2.5 FRACTIONATION OF SERUM LIPOPROTEINS USING POLYANIONS

This was performed using the method of Burstein et al. (1970) with either sodium phosphotungstate-magnesium chloride, or manganese chloride-dextran sulphate as precipitant.

2.5.1 Fractionation using sodium phosphotungstate and magnesium chloride

Procedure: 400 µl of 4% (w/v) sodium phosphotungstate and 100 µl of 2 mol/l magnesium chloride were added to 4 ml of serum. The final concentration of phosphotungstate (0.36% (w/v)) and magnesium chloride (44 mmol/l) caused immediate selective precipitation of both LDL and VLDL. The precipitate was removed by centrifugation for 10 min at 6000 x g, washed with 0.9% (w/v) NaCl containing 0.36% (w/v) phosphotungstate and 44 mmol/l magnesium chloride, and redissolved in 4 ml of 0.9% (w/v) NaCl.

1.8 ml of 4% (w/v) sodium phosphotungstate was added to 2.2 ml of the LDL- and VLDL-free supernatant (final concentrations: sodium phosphotungstate 2% (w/v), magnesium chloride 25 mmol/l). The precipitate, consisting largely of γ-globulin was removed by centrifugation for 10 min at 6000 x g and

discarded. 400 µl of 2 mol/l magnesium chloride was added to the supernatant (final concentrations: sodium phosphotungstate 1.8% (w/v), magnesium chloride 200 mmol/l). After 2 h, when precipitation of the HDL was complete the mixture was centrifuged for 30 min at 20 000 x g. The precipitate was washed in 0.9% (w/v) NaCl containing 2% (w/v) sodium phosphotungstate and 200 mmol/l magnesium chloride, and redissolved in 2.2 ml of 0.9% (w/v) NaCl. Enzyme activities were determined on all of the fractions.

2.5.2 Fractionation using dextran sulphate and manganese chloride

20 µl of 10% (w/v) dextran sulphate and 200 µl of 1 mol/l manganese chloride were added to 4 ml of serum. At these final concentrations (dextran sulphate 0.047% (w/v), manganese chloride 47 mmol/l) the LDL and VLDL were completely and selectively precipitated. The precipitate of LDL and VLDL was removed by centrifugation for 10 min at 6000 X g and washed with 0.9% (w/v) NaCl containing 0.047% (w/v) dextran sulphate and 47 mmol/l manganese chloride. The LDL and VLDL precipitate was then redissolved in 4 ml of 0.9%. (w/v) NaCl.

300 µl of 1 mol/l manganese chloride and 120 µl of 10% dextran sulphate were added to 1.55 ml of the LDL-and VLDL-free supernatant (final concentrations: manganese chloride 190 mmol/l, dextran sulphate 0.65% (w/v)). Precipitation of the HDL fraction began at once and was complete within 2 h. The mixture was centrifuged for 30 min at 20 000 x g, the supernatant collected and the HDL precipitate washed with 0.9% (w/v) NaCl containing 190 mmol/l manganese chloride and 0.65% (w/v) dextran sulphate. The HDL precipitate was redissolved in 1.55 ml of 0.9% (w/v) NaCl. Enzyme activities were determined on all of the fractions and the solutions kept for further study.

2.5.3 Lipoprotein electrophoresis

This was performed both upon sera and on the lipoprotein fractions obtained after polyanion precipitation on 1% (w/v) agarose using the Corning Universal electrophoresis apparatus. 1 to 5 µl of sample was added to the prepared wells

in the gel (Universal Electrophoresis Film) and electrophoresis performed for 35 min at 90 V using 50 mmol/l barbitone buffer, pH 8.6, (Martin and Franglen, 1954).

The lipoprotein fractions were made visible by staining for 5 min with a 0.2 g/l solution of Fat Red 7B in methanol-water (80/20, v/v) then cleared in an equal volume of methanol-water.

2.5.4 Validation of the lipoprotein fractionation method

This was done by performing lipoprotein electrophoresis (section 2.5.3) upon the various fractions obtained after treatment of serum with polyanions. The fractions were; i) whole serum; ii) serum after precipitation of LDL and VLDL; iii) serum after precipitation of HDL, LDL and VLDL; iv) LDL and VLDL (β lipoprotein); v) HDL (α-lipoprotein). Figure 2.7 confirms that the various lipoprotein fractions were precipitated as described in sections 2.5.1 and 2.5.2.

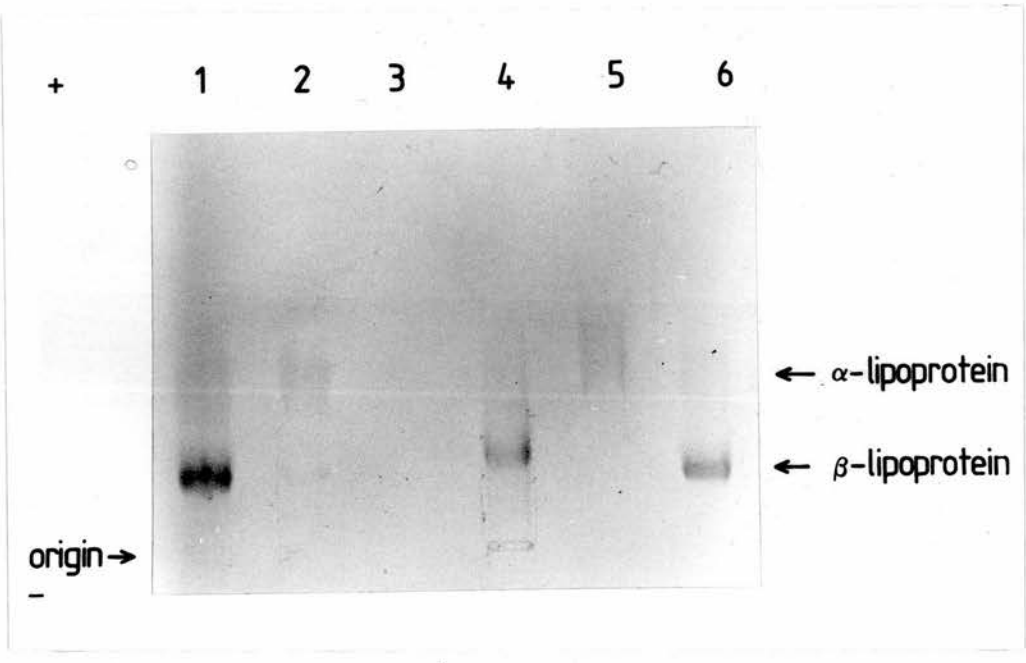
2.6 IMMUNOELECTROPHORESIS

This was carried out using a modification (Seidel et al., 1970) of the Scheidegger technique (1955).

Preparation of gel: A 1% (w/v) solution of agar was prepared by heating 1 g of agar in 100 ml of barbitone buffer, pH 8.6, in a boiling water bath for 60 min. When the agar had completely dissolved, the solution was cooled to 60°C ready for use.

The glass plates, 8.4 x 9.4 cm were cleaned with ethanol, pre-warmed in an oven and placed on a level surface. 14 ml of warm fluid agar was pipetted on to each plate and when the gels had formed, the plates were placed in a refrigerator for 30 min to ensure firmness. Five wells, each 2 mm in diameter and spaced 7.5 mm apart, were cut starting 4 cm from the anodal end of each plate. Each well was filled with 4 µl of sample.

Figure 2.7 Electrophoresis on agarose gel of whole serum and the fractions obtained after polyanion precipitation, stained for lipid. 1, whole serum; 2, supernatant after precipitation of LDL plus VLDL; 3, supernatant after precipitation of LDL plus VLDL, and HDL; 4, LDL plus VLDL; 5, HDL; 6, whole serum.



Electrophoresis: The plates were placed on the cooling plate, with a layer of water between them and the cooling plate, to provide thermal conduction. The wicks were placed on the gels to cover 1 cm of both the anodal and cathodal ends. Electrophoresis was performed for 2 h at 200 V.

Immunoprecipitation: Troughs 1 mm wide and 3 cm long were cut between the wells, filled with the appropriate antiserum and left to diffuse overnight at room temperature in a moist chamber to form any precipitin arcs. The gels were washed for 2 days in 0.9% (w/v) NaCl and stained for protein by soaking in 1% (w/v) naphthalene black dissolved in glacial acetic acid-methanol (10/90, (w/v)) for 60 s, and destained in glacial acetic acid-methanol (10/90, (w/v)) for 24 h.

Alternatively the precipitin arcs were stained for γGT activity using the method described in section 2.4.1.

2.7 MEASUREMENT OF BILE SALT CONCENTRATIONS IN BILE AND SERA

The primary bile salts, cholate and chenodeoxycholate, are present in serum and bile as either their taurine- or glycine-conjugates. In this study both were measured as their total conjugates by specific radio-immunoassays (Beckett et al., 1978; Beckett et al., 1979).

Reagents

Stripped serum: Bile salt-free serum was prepared by mixing 1 litre of pooled normal human serum with 1 litre of Amberlite XAD2 resin. The mixture was then stirred for 1 h at room temperature and left overnight at 4°C. The mixture was filtered and the serum kept for further use.

Working buffer: This contained disodium hydrogen orthophosphate (85 mmol/l), sodium dihydrogen orthophosphate (45 mmol/l), sodium azide (15 mmol/l) and sodium chloride (16 mmol/l) adjusted to pH 7.2 with 1 mol/l sodium hydroxide. 750 ml of stripped serum was added to 4250 ml of this buffer and the mixture stored at room temperature.

Wash solution: Ammonium sulphate (270 g/l).

Tracers: These consisted of ^{125}I- glycocholate and ^{125}I-glycochenodeoxycholate - 250 µCi/10 ml. These had been prepared by the iodination of histamine-glycocholate and histamine-glycochenodeoxycholate.

Working reagents: These were prepared by mixing either 25 µl of antiserum to glycocholate or 50 µl of antiserum to glycochenodeoxycholate with 30 µl of the appropriate tracer and 10 ml of working buffer.

Standard curve: A 1 mmol/l solution of both bile salts was prepared in absolute methanol. From this stock solution, standard curves were prepared by diluting the stock standard with working buffer. The standard values were 0, 0.5, 1.0, 2.0, 4.0, 6.0, 12.0 µmol/l.

Procedure

10 µl of sample was diluted with 200 µl of working reagent, mixed and incubated for at least 2 h at room temperature. 3 ml of wash solution was added to precipitate the bound fraction, mixed, and the mixture centrifuged at 3000 rpm at $4^{o}C$ for 15 min. The tubes were then inverted to discard the supernatant and the bound fraction counted in a Nuclear Enterprises 1600 γ counter.

The standard curve was fitted to a non-linear 4 parameter log-logistic model (Healy, 1972) using an iterative least-squares technique on a Hewlett-Packard 9821 calculator.

The coefficients of variation for both methods following repeat analysis of a serum pool 20 times, were 7.0% within-batch and 8.0% between-batch at a mean bile salt concentration of 3.0 µmol/l.

Sera with high bile salt concentrations were diluted 1/10 or 1/100 with stripped serum. Bile samples were always diluted 1/1000 with stripped serum prior to assay.

Chapter 3

PHYSICAL PROPERTIES OF γ-GLUTAMYLTRANSFERASE IN HUMAN BILE

Studies using gel chromatography on Sephadex G200 or similar material appear to show that γGT in human bile is present mainly as a high M_r form (Wenham et al., 1978a; Crofton and Smith, 1981a). However, if sodium deoxycholate is included in the chromatography buffer, a low M_r form of γGT is recovered in the eluate (Huseby, 1978). This suggests that γGT may be present in human bile in a low M_r form and that the high M_r form obtained by gel chromatography may merely represent an artefact caused by aggregation of the enzyme in aqueous media in which no detergent, i.e. bile salt, is present. The normal concentration of total bile salts in hepatic bile ranges from 3 to 45 mmol/l (Scharschmidt, 1982). Their critical micellar concentration is estimated to be 0.9 to 2.2 mmol/l, and so most will be present in micellar form (Tamesue and Juniper, 1967). However, bile salts are normally present in bile as conjugates of glycine or of taurine and the conjugates of cholate and of chenodeoxycholate are present in greater concentrations than those of deoxycholate (Sjovall, 1960; Heaton, 1972). The detergent properties of the bile salts, as measured by their rate of dissolution of cholesterol monohydrate, increase inversely with their polarity, and so decrease in the order deoxycholate, chenodeoxycholate, cholate (Carey, 1982). Experiments are described in this chapter involving the bile salt sodium deoxycholate that are designed to test the hypothesis that high M_r γGT is in fact an artefact of reaggregation. In order to mimic physiological conditions in bile more closely, the effects of glycocholate and glycochenodeoxycholate are also studied.

Leucine aminopeptidase and alkaline phosphatase have also been assumed to be present in native bile as high M_r forms since, after exclusion chromatography on Sephadex G200 or Sepharose 6B, they also elute mainly in the void volume (Price et al., 1972; Crofton and Smith, 1981a). These enzymes have also been included in the study to see whether they may be present in bile in forms comparable to those of γGT.

3.1 SAMPLES USED IN THE STUDY

Twelve samples of hepatic bile were obtained from patients with percutaneous or 'T' tube drainage of the common bile duct. The ranges of enzyme activities were as follows; γGT, 100-1540 U/L; LAP 22-126 U/L; ALP, 75-1150 U/L.

3.2 EFFECT OF PAPAIN TREATMENT ON γGT IN HUMAN BILE

Papain (EC 3.4.22.2) is a non-specific proteolytic enzyme obtained from papaya latex. Unlike the serine proteases, trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) it is insensitive to inhibition by di-isopropylfluorophosphate and depends upon a highly reactive sulphydryl group for its activity (Dixon and Webb, 1979). It has been shown to release LAP almost completely from rat liver membrane (Emmelot et al., 1968), and more recently, to convert γGT in bile to a lower M_r form (Huseby, 1978). A study of the effect of papain on γGT in human bile therefore seemed worthwhile in an attempt to gain further insight about γGT in hepatic bile.

Samples of bile were incubated with papain (1g/10g protein) overnight at 20°C in the presence of 100 mmol/l cysteine. Preliminary experiments were performed to determine whether papain had any effect per se on γGT activity. The results presented in Table 3.1 show that papain treatment of bile resulted in slight decrease in the γGT activity.

Table 3.1

γGT activity after digestion of human bile with papain

Sample	Percentage initial activity
Bile A	93
Bile B	78
Bile C	94

3.3 EFFECT OF BILE SALTS ON γGT ACTIVITY IN HUMAN BILE

Prior to gel chromatography or electrophoresis, bile salts, equal to the concentration used in the appropriate buffer, were added to the bile. The bile was assayed for γGT activity before and immediately after the addition, to see whether bile salts also had an effect upon γGT activity *per se*. It was then stored for 12 h prior to further investigation. Bile salts appeared to have no effect upon total γGT activity (Table 3.2).

Table 3.2

Effects of different concentrations of added bile salts on γGT activity in bile

	Percentage initial γGT activity
added glycocholate (mmol/l)	
0*	100
5	100
10	102
15	102
20	104
added glycochenodeoxycholate (mmol/l)	
0*	100
1	95
3	94
5	94
10	93

* The endogenous concentration of conjugated cholate was 2.7 mmol/l and of conjugated chenodeoxycholate 0.5 mmol/l.

3.4 GEL FILTRATION CHROMATOGRAPHY ON SEPHADEX G200

3.4.1 General nomenclature used throughout this chapter

A fairly clear pattern of results emerged following these investigations. In order to simplify the description, the peaks were named separately in order of their elution from the gel column:

Peak 1 (γGT), Peak 1 (LAP), Peak 1 (ALP): void volume for all enzymes.

Peak 2 (γGT): a variable peak with estimated $\underline{M}_r$ in the range 300 000 - 600 000 (this wide range may well represent partial dissociation of enzyme complexes whilst running on the column).

Peak 2T (γGT), Peak 2T (LAP), Peak 2T (ALP): major intermediate $\underline{M}_r$ peaks eluting when 0.1% (w/v) Triton X-100 is present in the elution buffer.

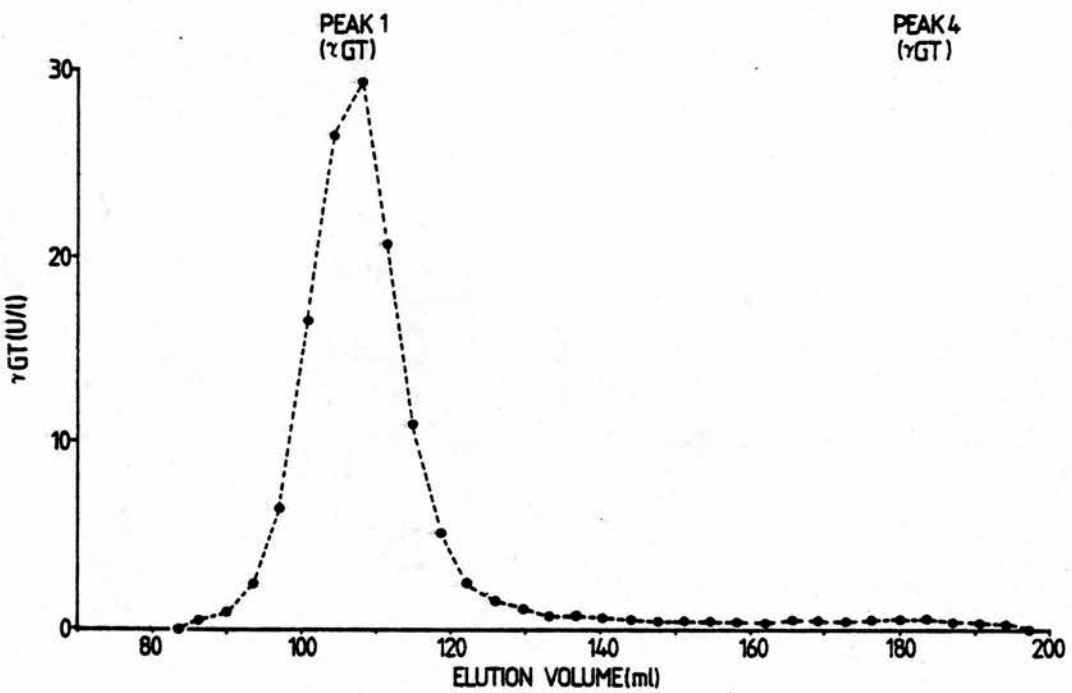
Peak 3 (γGT), Peak 3 (LAP), Peak 3 (ALP): major low $\underline{M}_r$ peaks eluting when bile salts are present in the eluting buffer. These peaks reaggregate in the absence of bile salts.

Peak 4 (γGT), Peak 4 (LAP), Peak 4 (ALP): minor peaks in bile, eluting after the corresponding Peak 3 and found in small amounts in native bile, and in large amounts (for γGT and LAP) in bile which has been previously treated with papain. These peaks do not undergo reaggregation in the absence of bile salts.

3.4.2 Gel chromatography performed in the absence of detergents

After gel chromatography of native bile, between 87 and 97% of γGT activity eluted in the void volume, with the remainder eluting as Peak 4 (γGT) (Fig. 3.1). The recovery of γGT activity ranged from 80 - 100%.

Figure 3.1 Elution profile of γGT in human bile following gel chromatography on Sephadex G200.



3.4.3 Gel chromatography performed in the presence of sodium deoxycholate

Bile was incubated with an equal volume of 25 mmol/l sodium deoxycholate for 1 h at room temperature and 1 - 2 ml of the mixture subjected to gel chromatography with varying concentrations of deoxycholate in the elution buffer. The main peak of γGT activity changed from Peak 1 (γGT), when there was no deoxycholate in the elution buffer, to Peak 3 (γGT), when the deoxycholate concentration was more than 5 mmol/l. However, at intermediate deoxycholate concentrations, a significant amount of activity was present as Peak 2 (γGT) with little or no activity in Peak 3 (γGT) (Fig. 3.2).

3.4.4 Gel chromatography performed in the presence of glycocholate and glycochenodeoxycholate

Gel chromatography with increasing concentrations of glycocholate in the elution buffer resulted in a decrease in the amount of γGT activity eluting in the void volume and the appearance of a fraction eluting as Peak 3 (γGT). Between 41 and 56% of γGT activity eluted as Peak 3 (γGT), when the elution buffer contained 20 mmol/l glycocholate (Fig. 3.3).

Similarly, when glycochenodeoxycholate was included in the elution buffer at increasing concentrations, the main peak of activity changed from Peak 1 (γGT) to Peak 3 (γGT). At a concentration of 5 mmol/l, 81 - 95% of γGT activity eluted as Peak 3 (γGT) (Fig. 3.4). At intermediate concentrations of glycochenodeoxycholate, significant amounts of γGT activity eluted as Peak 2 (γGT), with little or no activity in Peak 3 (γGT).

3.4.5 Gel chromatography performed in the presence of Triton X-100

Triton X-100 (polyoxyethylene glycol (9-10) p-t-octyl-phenol) is a fairly mild non-ionic detergent and has been used to solubilize membrane proteins (Sohn and Marinetti, 1974; Helenius and Simons, 1975). More recently, high $\underline{M}_r$ ALP in human bile has been converted by Triton X-100 to a form of lower $\underline{M}_r$

Figure 3.2 Gel chromatography on Sephadex G200 of γGT in deoxycholate-treated hepatic bile, with increasing concentrations of deoxycholate in the eluting buffer. ○ , no deoxycholate; ■ , 1.2 mmol/l deoxycholate; ● , 7 mmol/l deoxycholate.

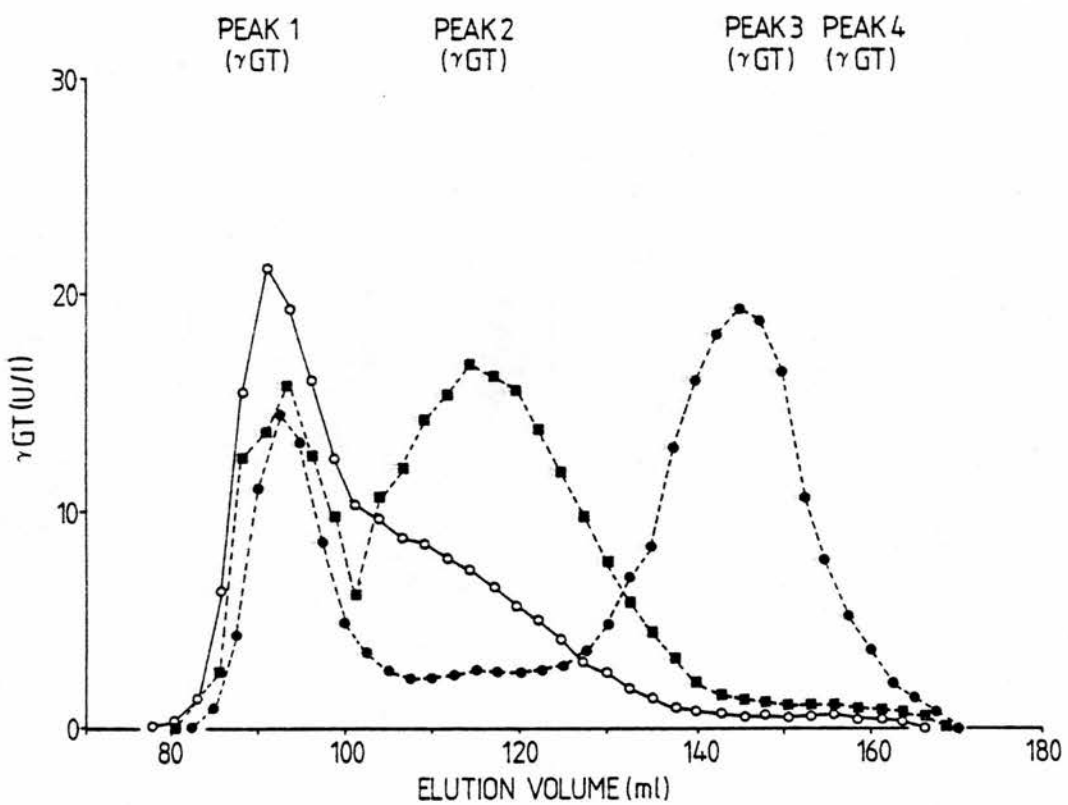


Figure 3.3 Gel chromatography on Sephadex G200 of γGT in human bile with increasing concentrations of glycocholate in the elution buffer. ○ , 5 mmol/l glychocholate; ■ , 10 mmol/l glychocholate; ● , 20 mmol/l glycocholate.

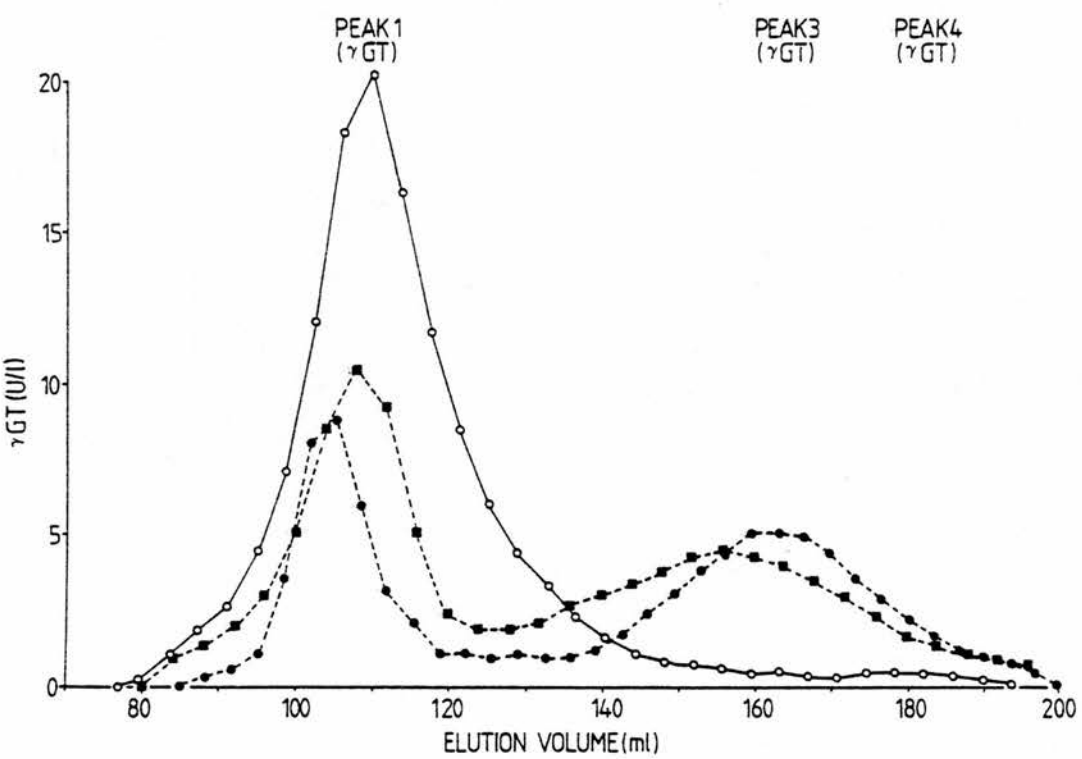
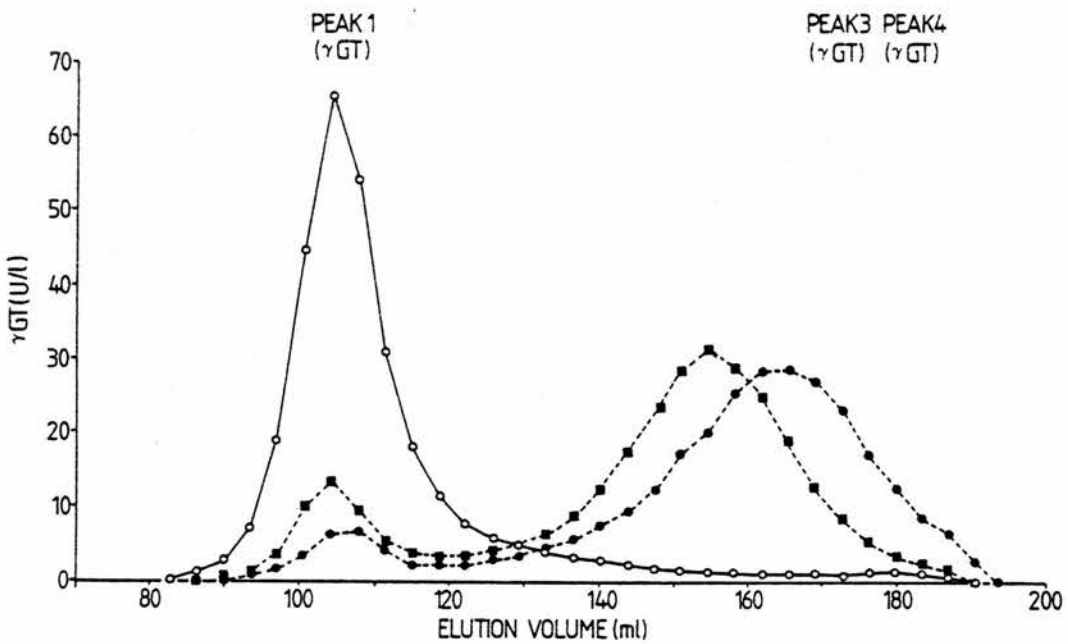


Figure 3.4 Elution profile of γGT following gel chromatography of human bile on Sephadex G200, with increasing concentrations of glycochenodeoxycholate in the elution buffer. ○ , 1 mmol/l glycochenodeoxycholate; ■ , 3 mmol/l glycochenodeoxycholate; ● , 5 mmol/l glycochenodeoxycholate.



(Crofton and Smith, 1981a). It therefore seemed worthwhile to study the effects of Triton X-100 on γGT in hepatic bile.

Two volumes of bile were mixed with 1 volume of 0.3% (w/v) Triton X-100 and stored at 4°C overnight. Gel chromatography of the treated bile (2 ml) with 0.1% (w/v) Triton X-100 in the elution buffer resulted in 90% of γGT activity eluting as Peak 2T (γGT). Small amounts of activity still eluted as Peak 1 (γGT) with the remainder as Peak 4 (γGT) (Fig. 3.5).

3.4.6 Gel chromatography of papain-treated bile

After papain treatment, gel chromatography in the absence of detergents resulted in a decrease in γGT activity eluting in the void volume, together with an increase in activity eluting as Peak 4 (γGT) (Fig. 3.6).

The estimated M_r of the various fractions obtained after gel chromatography in the presence and absence of bile salts is shown in Table 3.3.

Table 3.3

Estimated M_r values of the γGT fractions obtained following gel chromatography

Fraction	Estimated M_r
Peak 1	> 600 000
Peak 2T	333 000
Peak 3	161 000*
Peak 4	115 000

* Mean value, range 157 000 - 165 000, due to between-column variation.

Figure 3.5 Elution profile of γGT after gel chromatography of human bile on Sephadex G200 with 0.1% (w/v) Triton X-100 in the elution buffer.

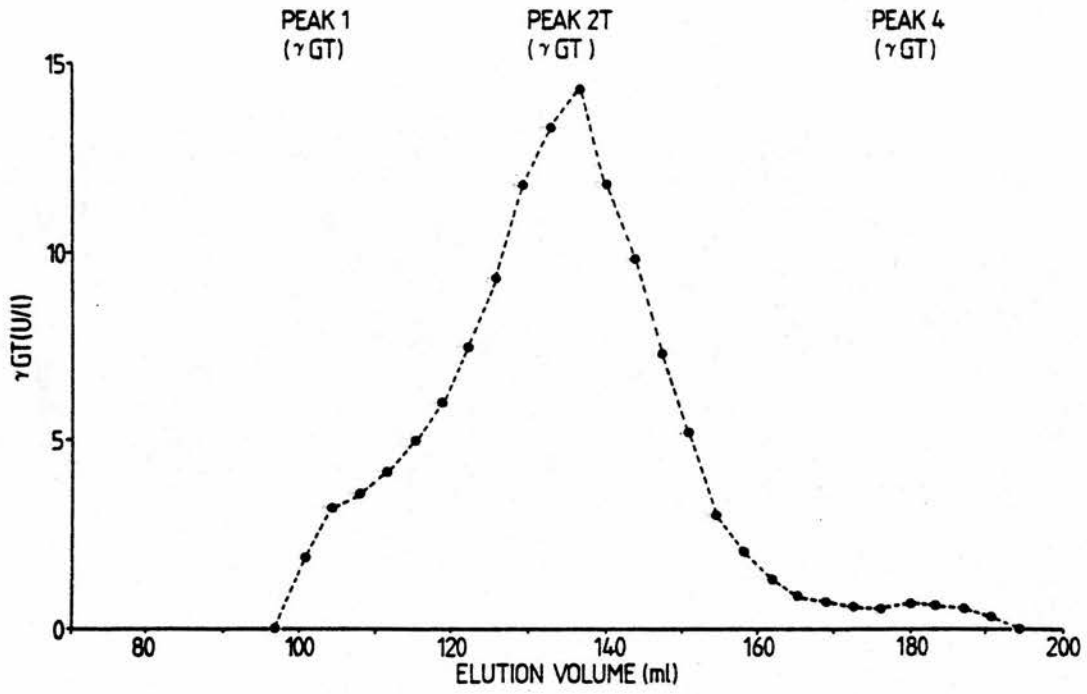
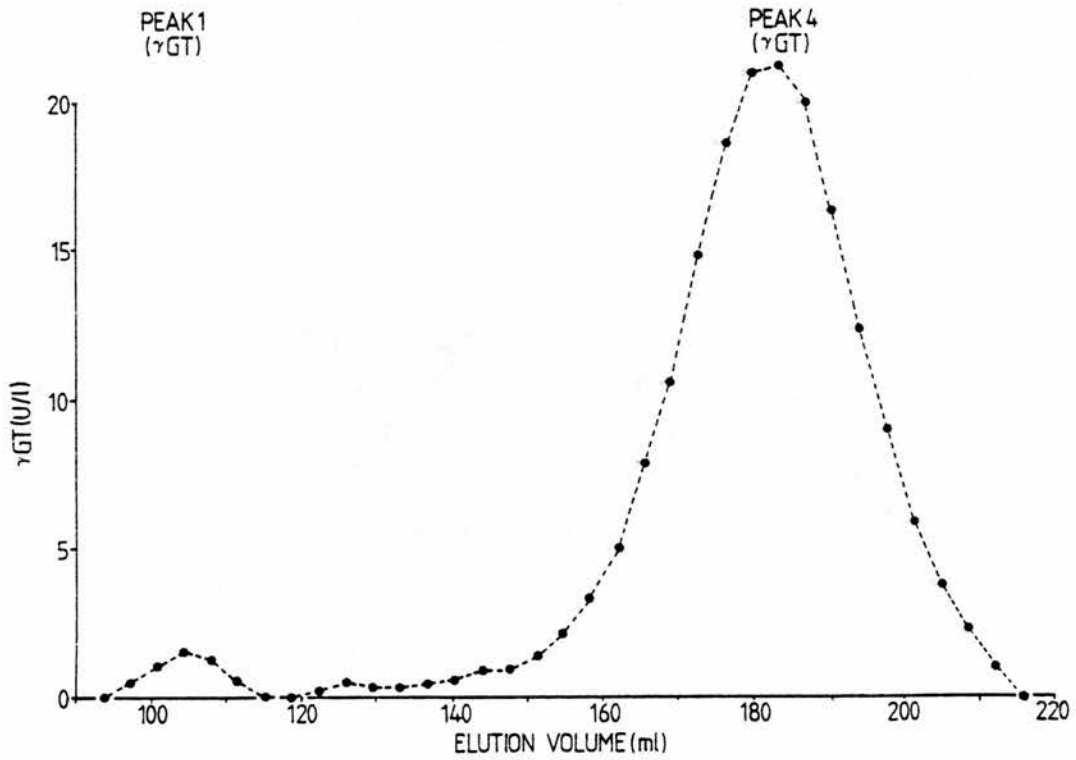


Figure 3.6 Sephadex G200 gel chromatography of papain-treated γGT present in hepatic bile.



3.5 7% POLYACRYLAMIDE GEL ELECTROPHORESIS

3.5.1 Electrophoresis in the absence of detergents

After electrophoresis, untreated bile showed a major band of activity at the origin (Band I (γGT)), with a small amount of activity present as a band with approximately 70 - 80% of the mobility of serum albumin (Band IV (γGT)) (Fig. 3.7). Occasionally a weak additional band of intermediate mobility was seen. Electrophoresis of concentrated pooled fractions corresponding to Peak 1 (γGT) and Peak 4 (γGT) obtained by gel chromatography, revealed that Peak 1 (γGT) gave rise to Band 1 (γGT), and that Band IV (γGT) had identical mobility to concentrated pooled fractions corresponding to Peak 4 (γGT) (Fig. 3.8).

3.5.2 Electrophoresis performed in the presence of detergents

Electrophoresis in 7% polyacrylamide gel, pre-equilibrated with the running buffer containing 5 mmol/l glycochenodeoxycholate or deoxycholate, showed the following pattern. Both native bile and Peak 3 (γGT), which was obtained by performing gel chromatography in the presence of bile salts, contained a main zone of activity (Band III (γGT)), which migrated just ahead of the zone due to Peak 4 (γGT) (Band IV (γGT)) (Fig. 3.9). This suggests that the Peak 3 form of γGT possesses a greater negative charge than the Peak 4 form.

When both the gel and running buffer had been equilibrated with 0.1% (w/v) Triton X-100, electrophoresis of Peak 2T (γGT), obtained after gel chromatography in the presence of 0.1% (w/v) Triton X-100, gave rise to a single band, the mobility of which was about 10% of that of Band IV (γGT) (Band IIT (γGT)) (Fig. 3.10).

Figure 3.7 7% polyacrylamide slab gel electrophoresis of hepatic bile stained for γGT activity. 1, bile A; 2, bile B.

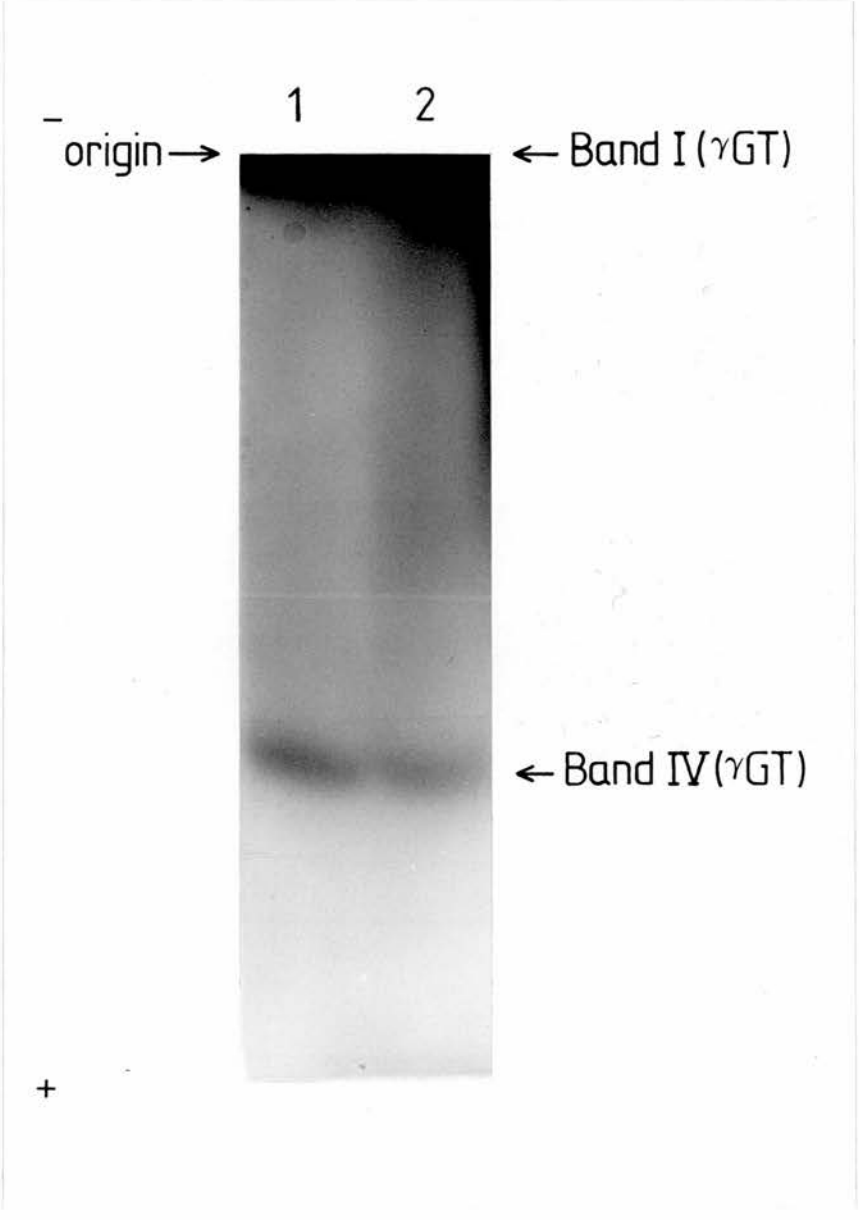


Figure 3.8 Electrophoresis on 7% polyacrylamide gel of hepatic bile and the fractions obtained after gel chromatography, stained for γGT. 1, 3, hepatic bile; 2, Peak 1 (γGT); 4, Peak 4 (γGT).

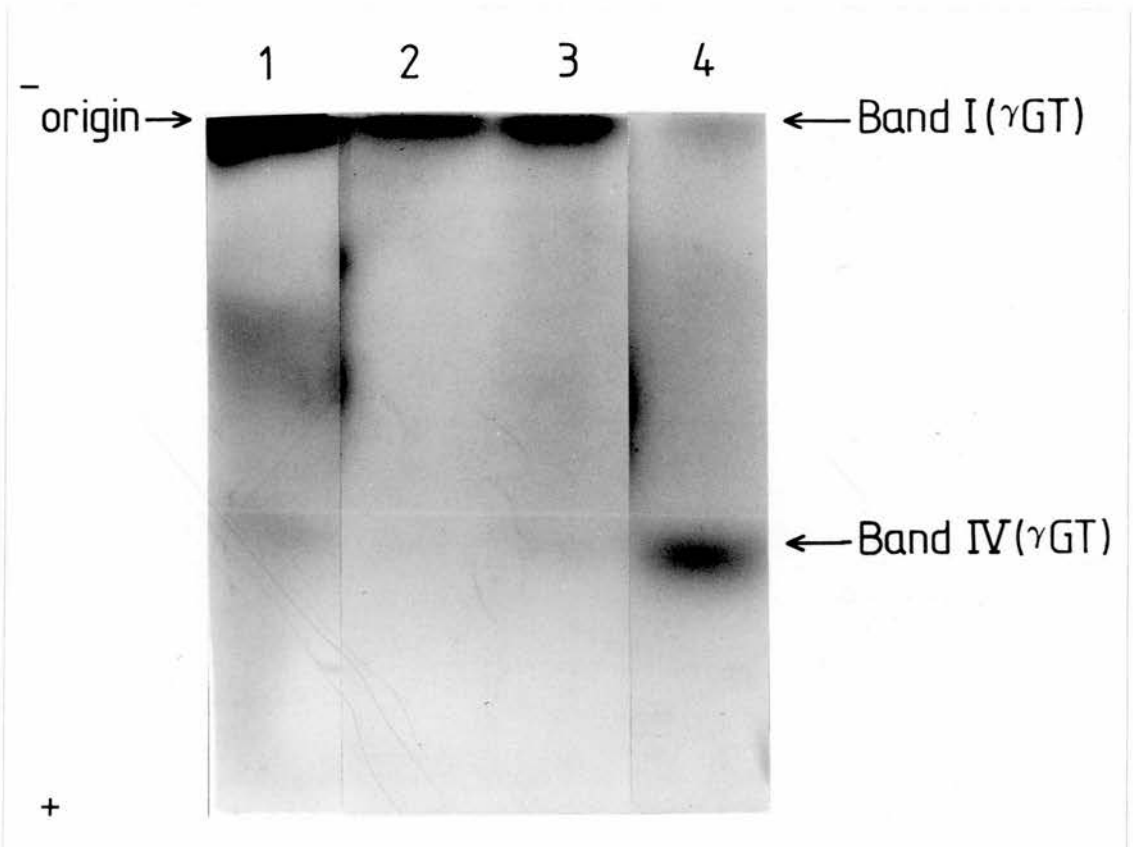


Figure 3.9 Polyacrylamide slab gel electrophoresis in 5 mmol/l glycochenodeoxycholate, stained for γGT activity. 1, bile A; 2, bile B; 3, bile C; 4, Peak 3 (γGT); 5, Peak 4 (γGT).

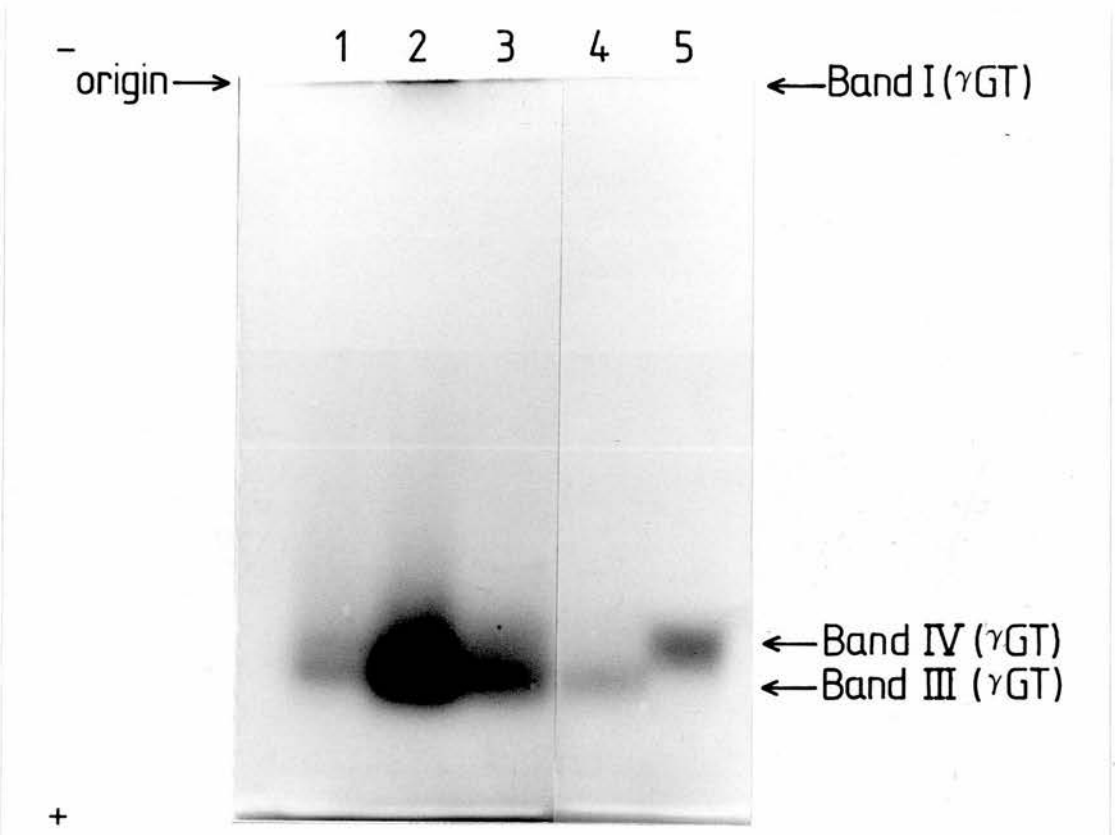
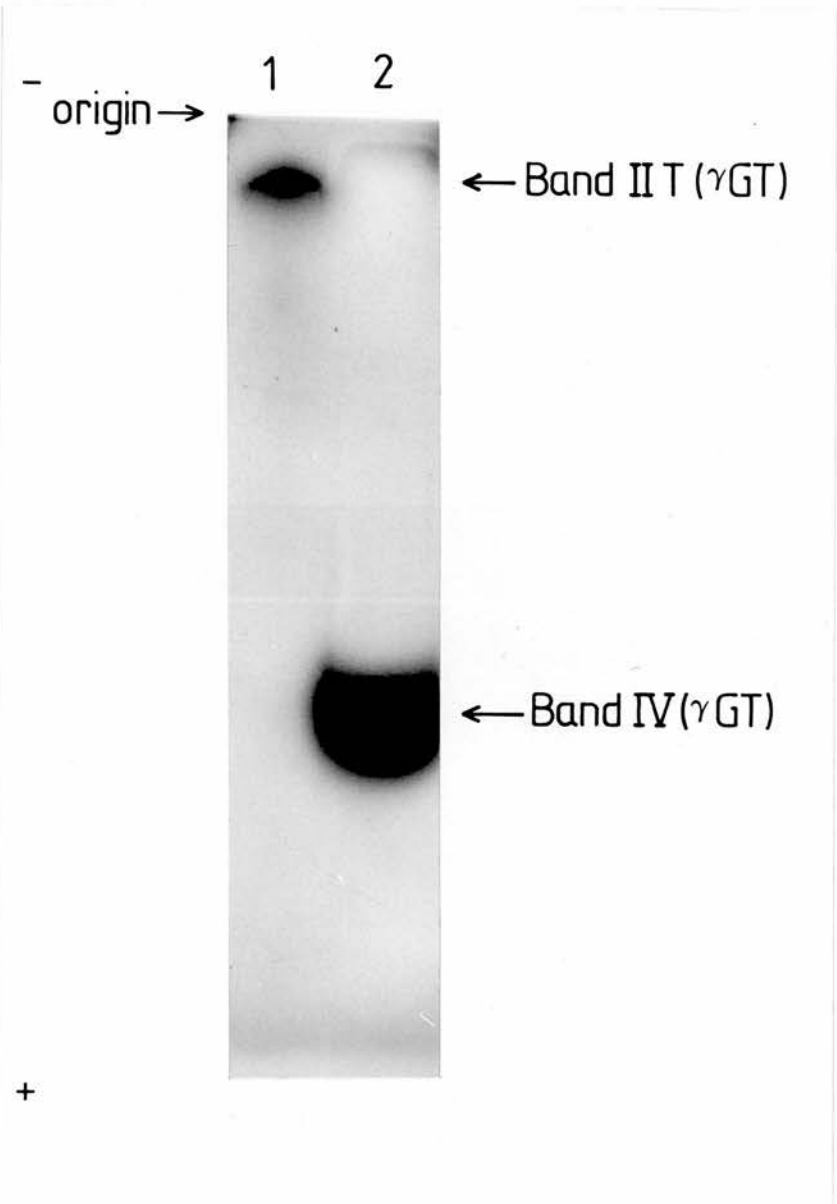


Figure 3.10 Polyacrylamide slab gel electrophoresis in 0.1% (w/v) Triton X-100 of the fractions obtained after gel chromatography. 1, Peak 2T (γGT); 2, Peak 4 (γGT).



3.6 POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS

The Peak 1 form of γGT had an $\underline{M}_r$ of greater than 1 000 000, whereas the Peak 3 and Peak 4 forms were both of low $\underline{M}_r$. Peak 4 (γGT), obtained from untreated bile, had a lower $\underline{M}_r$ than Peak 3 (γGT), obtained from bile treated with either deoxycholate or glycochenodeoxycholate. The estimated $\underline{M}_r$ values of the various fractions are shown in Table 3.4.

Table 3.4

Estimated $\underline{M}_r$ values of the various γGT fractions obtained from hepatic bile as determined by polyacrylamide gradient gel electrophoresis

Fraction	Estimated $\underline{M}_r$	
	Mean	Range
Peak 1	> 1000 000	*
Peak 3	130 000	125 000 - 135 000
Peak 4	104 000	98 000 - 110 000

* Not estimable

3.7 STUDIES ON THE FRACTIONS OBTAINED BY GEL CHROMATOGRAPHY

3.7.1 Studies on peak 3 (γGT)

The experiments were designed to determine if Peak 3 (γGT) reaggregated in the absence of bile salts.

A concentrated pool of Peak 3 (γGT), obtained by performing gel chromatography in the presence of 5 mmol/l deoxycholate, was

rechromatographed a) in the presence of 5 mmol/l deoxycholate and b) in the absence of bile salts. The elution profiles (Fig. 3.11) show that, in the presence of deoxycholate, Peak 3 (γGT) retains its low $\underline{M}_r$ form, whereas chromatography in the absence of deoxycholate causes complete reaggregation of the γGT into a form with $\underline{M}_r$ greater than 600 000.

3.7.2 Studies on Peak 4 (γGT)

These experiments were designed to determine if Peak 4 (γGT), when added to bile, was able to form high $\underline{M}_r$ complexes of γGT.

Samples of bile, containing 0.24 U of γGT activity, were added to 0.5 ml of concentrated Peak 4 (γGT) eluate, also containing 0.24 U of γGT activity. The volumes were made up to 2 ml with Tris-HCl buffer, pH 8.0, and the solutions incubated for 6 h and 30 h, before being subjected to gel chromatography in the absence of bile salts. The elution profiles (Fig. 3.12) show that the incubation did not alter the chromatographic behaviour of Peak 4 (γGT).

3.8 INVESTIGATION INTO POSSIBLE BILE SALT BINDING TO γGT

This was carried out in an attempt to demonstrate if bile salt binding to γGT could account for the differences in size and charge between the γGT in Peaks 3 and Peak 4.

3.8.1 Radioactive counting

This was performed in a Packard Tri-Carb liquid scintillation spectrometer (model 3255) using a toluene: Triton X-100 (2:1 (v/v)) based scintillant containing 2,5-diphenyl oxazole, 4 g/l and 1, 4 - di (2-(5-phenyl oxazolyl)) - benzene, 0.2 g/l. 5 ml of scintillant was mixed with 100 µl of aqueous sample in polyethylene vials and counted with a standard deviation of less than 1%. Samples were corrected for quenching by the channels ratio method. The

Figure 3.11 Rechromatography on Sephadex G200 of Peak 3 (γGT) in the presence ● , or absence ○ , of 5 mmol/l deoxycholate.

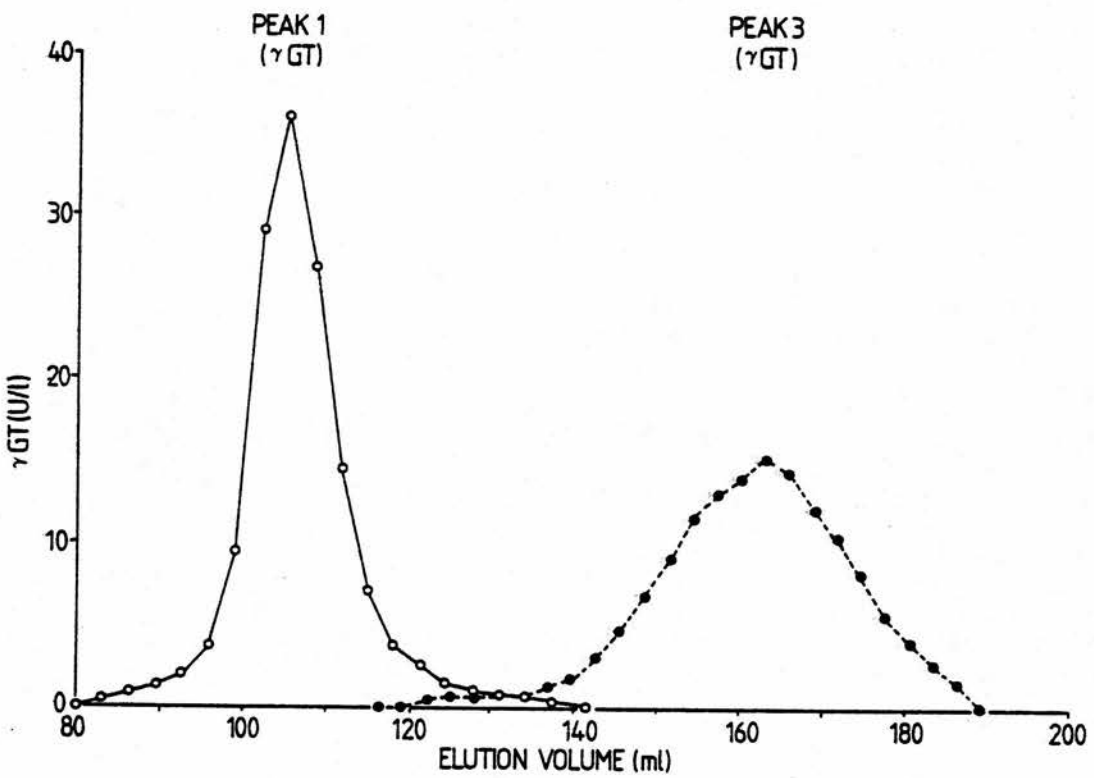
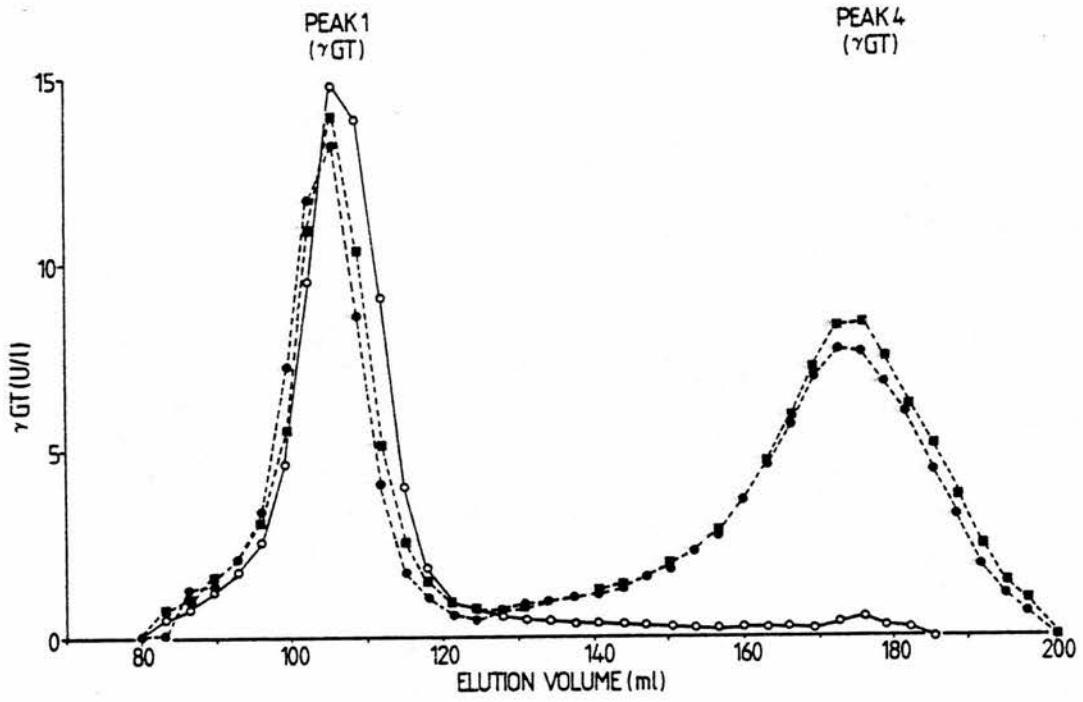


Figure 3.12 Gel chromatography on Sephadex G200 of human bile before and after incubation with Peak 4 (γGT). ○ , before; ■ , after 6 hours incubation; ● , after 36 hours incubation.



counting efficiency was determined by counting known amounts of [^{14}C] deoxycholate in hexadecane and was found to be 67%.

3.8.2 Investigation into bile salt binding under non-equilibrium conditions

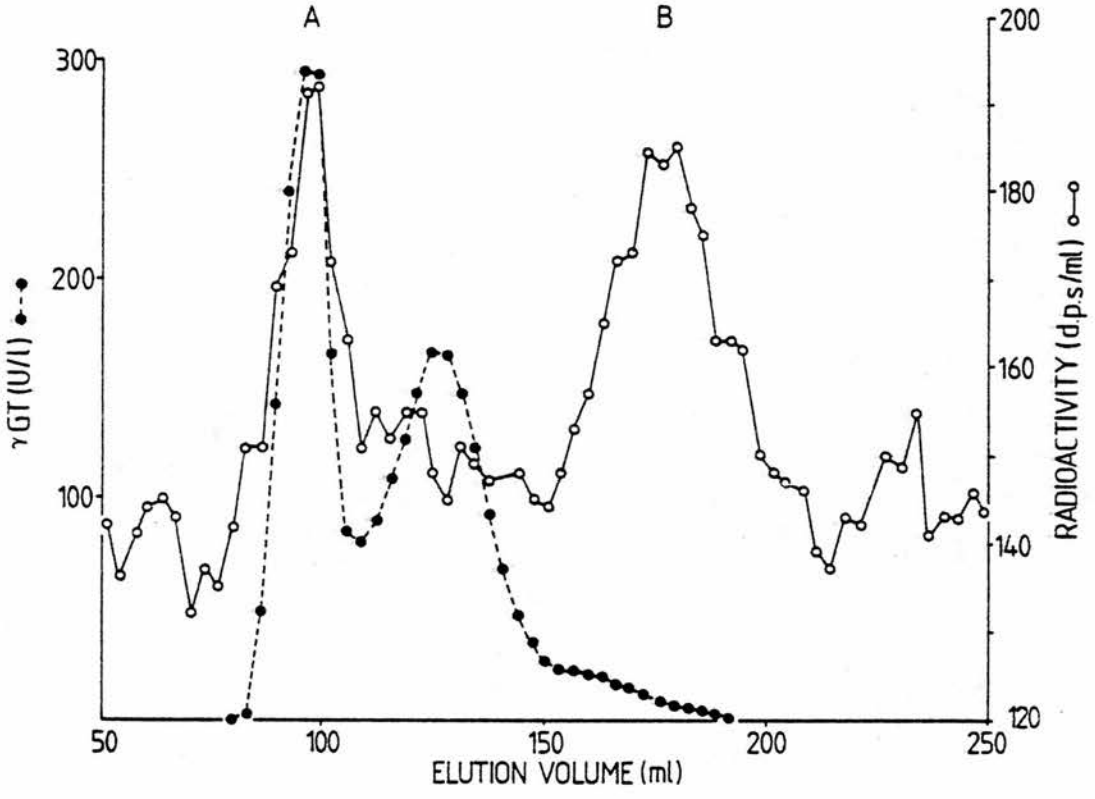
This was studied using gel chromatography. Neat bile (2 ml) was incubated for 1 h at room temperature with 5 µl of a solution containing 1µCi [^{14}C] deoxycholate (19.2 nmol). This mixture was then chromatographed on Sephadex G200 and radioactivity and γGT activity measured in the eluate. Radioactivity was only present in the salt volume of the column and was not associated with any of the peaks of γGT activity.

3.8.3 Investigation into bile salt binding under equilibrum conditions

To investigate the possibility that any γGT-deoxycholate complexes had dissociated during chromatography, deoxycholate binding was also studied using an equilibrum method. Equilibrum chromatography, introduced by Hummel and Dreyer (1962), avoids the problem of dissociation since the column is eluted with a constant concentration of ligand. The ligand-protein complex is, therefore, constantly surrounded by free ligand during chromatography. In order to increase the amount of γGT protein available for possible binding, bile was concentrated approximately 20 times, prior to use, using a Minicon macro solution concentrator (Amicon, London, U.K.).

2 ml of concentrated bile was incubated for 1 h with 1µCi [^{14}C] deoxycholate. Column chromatography of the mixture on Sephadex G200, which had been pre-equilibrated with [^{14}C] deoxycholate (5µCi/l), resulted in three peaks of radioactivity. The first, Peak A, co-eluted with the void volume and contained the majority of γGT activity. The second, Peak B, eluted at 175 ml, corresponding to an estimated $\underline{M}_r$ of about 70 000, and the third, Peak C, eluted with the salt volume; there was no γGT activity associated with these last two peaks (Fig. 3.13). The ratio of radioactivity in Peak B to that in Peak A was 1.45:1.

Figure 3.13 Elution profile of γGT and [^{14}C] deoxycholate after gel chromatography of hepatic bile on a column of Sephadex G200, equilibrated with 5 μ Ci/l (99 nmol/l) of deoxycholate.



This experiment was repeated using bile which had previously been treated with papain. The elution pattern of radioactivity obtained was similar to that above apart from the fact that a relatively greater amount of radioactivity appeared in Peak B (ratio of Peak B to Peak A was 1.83:1, (Fig. 3.14). The total amount of radioactivity in Peak B was approximately the same in both experiments.

3.9 ULTRACENTRIFUGATION

If γGT in native bile is present as a low M_r form, then significant quantities of γGT activity should remain in the supernatant following centrifugation at high speed. In order to determine if this is so, the following experiments were carried out.

Samples of bile (before and after treatment with bile salts), (10 ml) were centrifuged at 2500 x g for 10 min. 8.5 ml of the supernatant was collected, the pellet was washed with 1 ml of 20 mmol/l Tris-HCl buffer, pH 8.0, and then resuspended in 2 ml of the same buffer. γGT activity was estimated on all fractions.

Over 92% of γGT activity was recovered in the supernatant following centrifugation at 2500 x g. After a further centrifugation of this supernatant at 150 000 x g for 1 h, the percentage of γGT activity recovered in the supernatant ranged from 25 - 75%. Addition of increasing concentrations of glycochenodeoxycholate to the bile prior to centrifugation resulted in more activity being recovered in the 150 000 x g supernatant (Fig. 3.15). The observed increase in recovered supernatant activity was greater in the samples from patients in whom bile had been draining for several days before the collection was made. These samples had a low concentration of bile salts due to depletion of the bile salt pool.

Figure 3.14 Elution profile of [^{14}C] deoxycholate and γGT following gel chromatography of papain-treated bile on Sephadex G200. The Sephadex column was equilibrated with 5 Ci/l 99 nmol/l) deoxycholate.

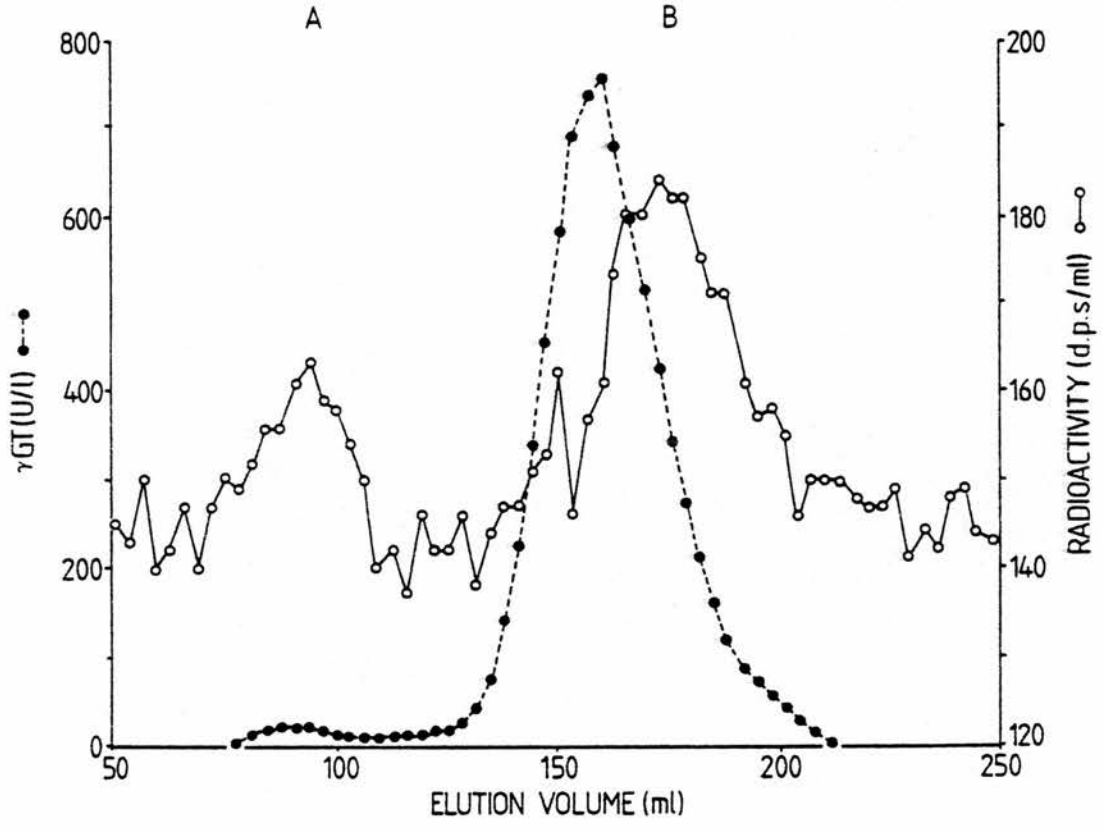
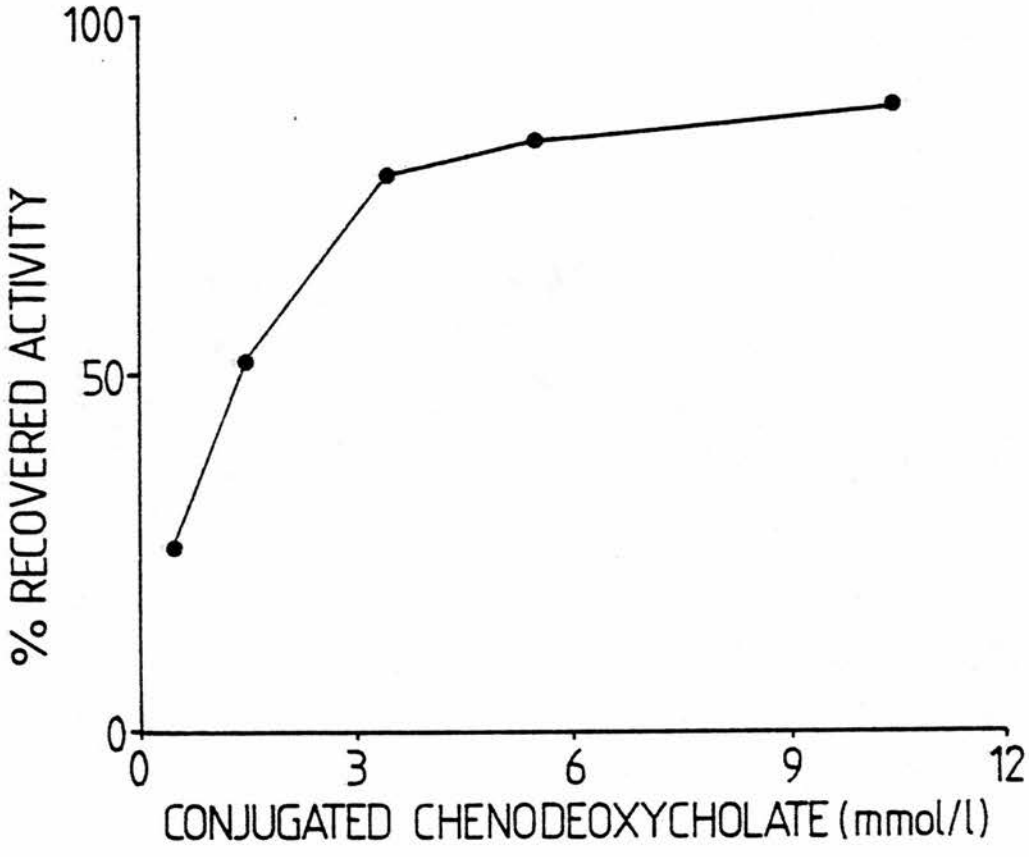


Figure 3.15 Percentage recovery of γGT activity in the supernatant following ultracentrifugation of hepatic bile at 150 000 x g, plotted against conjugated chenodeoxycholate concentration.



3.10 STUDIES ON LEUCINE AMINOPEPTIDASE AND ALKALINE PHOSPHATASE

3.10.1 Effect of papain treatment

Digestion of hepatic bile with papain had a variable effect upon LAP activity, whereas ALP activity was destroyed following this treatment (Table 3.5).

Table 3.5

Enzyme activities after digestion of human bile with papain

Sample	Percentage initial activity	
	LAP	ALP
Bile A	108	3
Bile B	74	0
Bile C	84	12

3.10.2 Effect of bile salts upon enzyme activities

Sodium glycocholate, at concentrations up to 20 mmol/l, appeared to stimulate ALP activity slightly, whereas sodium glycochenodeoxycholate appeared to have no effect. Both bile salts significantly inhibited LAP activity (Table 3.6).

Table 3.6

Enzyme activities following addition of bile salts to human bile

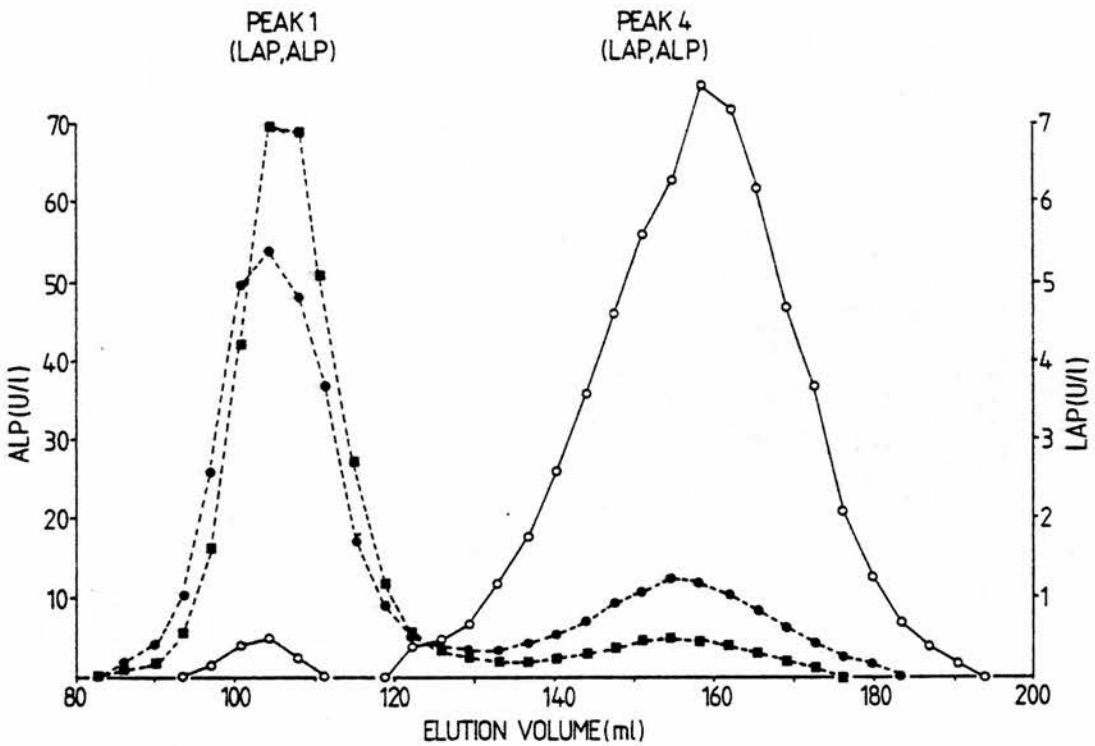
	Percentage initial enzyme activity	
	LAP	ALP
Added glycocholate (mmol/l)		
0*	100	100
5	86	117
10	73	131
15	59	131
20	49	139
Added glycochenodeoxycholate (mmol/l)		
0*	100	100
1	83	92
3	68	97
5	59	108
10	42	110

* The endogenous concentration of conjugated cholate was 2.7 mmol/l and of conjugated chenodeoxycholate 0.5 mmol/l.

3.10.3 Gel chromatography performed in the absence of bile salts

Both LAP and ALP eluted mainly as void volume peaks (Fig. 3.16), comprising 61 - 76% and 83 - 98% respectively of total recovered activity with the remainder eluting as Peak 4 (LAP) and Peak 4 (ALP). Recovery of LAP ranged from 80 - 100% and of ALP from 100 - 220%. The high recovery for ALP may possibly be due to low $\underline{M}_r$ inhibitors of ALP present in native bile but which become separated from the enzyme as it passes through the gel column.

Figure 3.16 Elution profiles of LAP and ALP in human bile following gel chromatography on Sephadex G200 before and after treatment with papain. ● , LAP before; ○ , LAP after; ■ , ALP before.



After papain treatment, gel chromatography in the absence of detergents resulted in a decrease in LAP activity eluting in the void volume, along with an increase in activity eluting as Peak 4 (LAP) (Fig. 3.16).

3.10.4 Gel chromatography performed in the presence of bile salts

Gel chromatography, with increasing concentrations of glycocholate in the elution buffer, resulted both in a decrease in the amount of ALP activity eluting in the void volume and in the appearance of a fraction eluting as Peak 3 (ALP). At a concentration of 20 mmol/l glycocholate, the proportion of ALP activity eluting as Peak 3 (ALP) ranged from 32 - 45%. Results for LAP could not be obtained because, at high concentrations of glycocholate, the enzyme was almost completely inhibited during its passage through the column.

When glycochenodeoxycholate was included in the elution buffer, at increasing concentrations, the main peak of activity for both LAP and ALP changed from Peak 1 to Peak 3. When the concentration was 5 mmol/l, 63 - 87% of LAP and 73 - 77% of ALP eluted as Peak 3 (Fig. 3.17).

3.10.5 Gel chromatography performed in the presence of Triton X-100

Bile, previously incubated with Triton X-100 (see section 3.4.5), was applied to a Sephadex G200 column and eluted with buffer also containing Triton X-100 (0.1% w/v). The majority of LAP (71%) and ALP (84%) eluted as peaks 2T (Fig. 3.18). Small amounts of activity (6 - 11%) still eluted as Peak 1 with the remainder as Peak 4.

The estimated $\underline{M}_r$ of the various fractions obtained after gel chromatography are listed in Table 3.7.

Figure 3.17 Elution of LAP and ALP following gel chromatography on Sephadex G200 containing 5 mmol/l glycochenodeoxycholate in the elution buffer. ● , LAP; ■ , ALP.

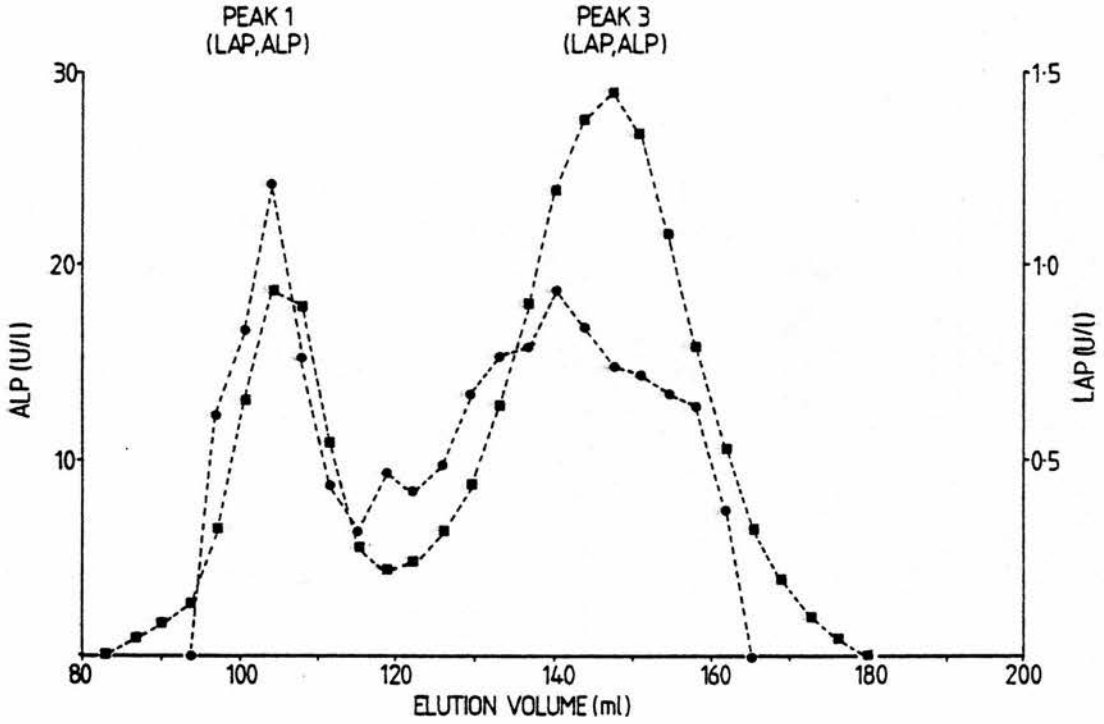


Figure 3.18 Gel chromatography of LAP and ALP in human bile on Sephadex G200 equilibrated with 0.1% (w/v) Triton X-100. ● , LAP; ■ , ALP.

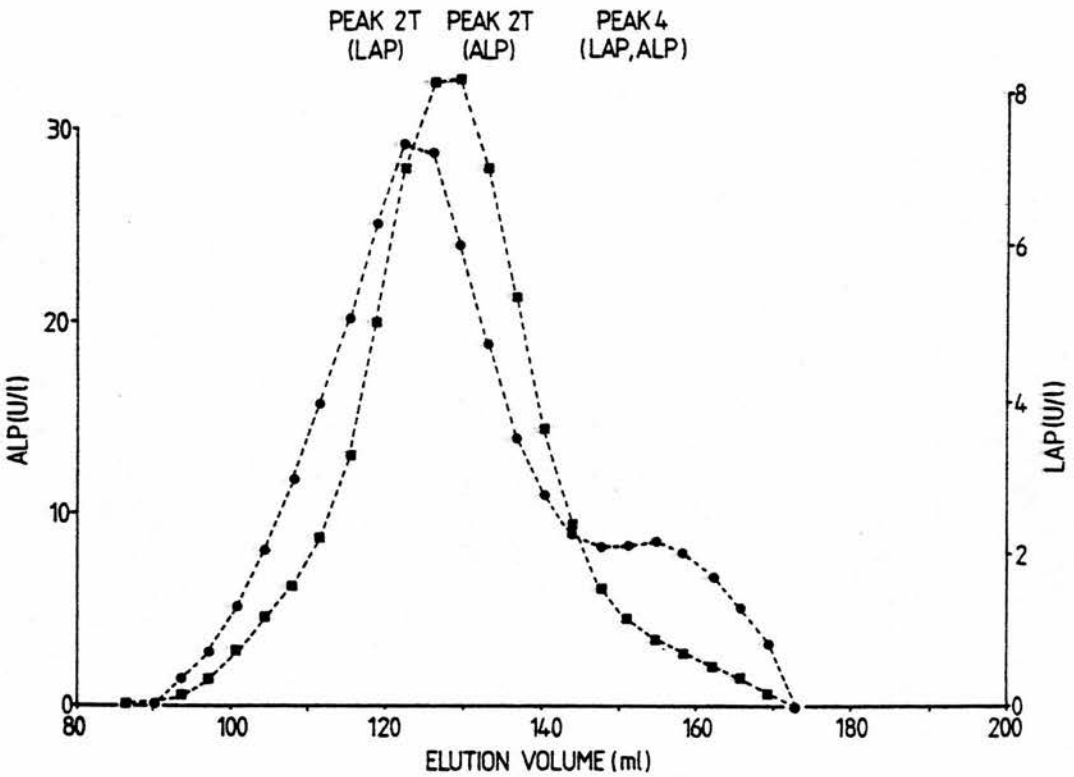


Table 3.7

Estimated M_r of the enzyme fractions obtained following gel chromatography

Fraction	Estimated M_r	
	LAP	ALP
Peak 1	>600 000	>600 000
Peak 2T	430 000	400 000
Peak 3	310 000	290 000
Peak 4	200 000	200 000

3.10.6 7% polyacrylamide gel electrophoresis

The electrophoresis of bile, of Peak 3 (ALP) and of Peak 4 (ALP) in 7% polyacrylamide gel, equilibrated in both the gel and running buffer with 5 mmol/l glychochenodeoxycholate, showed a single fast band of identical mobility in all three samples (Fig. 3.19). The peak 4 (ALP) was obtained after performing gel chromatography of serum from a patient with liver disease. Results for LAP could not be obtained owing to its inhibition by bile salts.

3.10.7 Polyacrylamide gradient gel electrophoresis

The results obtained for alkaline phosphatase were analogous to those obtained for γGT. Thus, the estimated M_r values for the Peak 4 forms of ALP in untreated bile, and in bile treated with papain, were less than those for the corresponding Peak 3 forms (Table 3.8). Again, results for LAP could not be obtained in the presence of bile salts because of inhibition.

Figure 3.19 Polyacrylamide slab gel eletcrophoresis, in the presence of 5 mmol/l glycochenodeoxycholate, of hepatic bile and the fractions obtained from gel chromatography, stained for ALP. 1, hepatic bile; 2, Peak 3 (ALP); 3, Peak 4 (ALP).

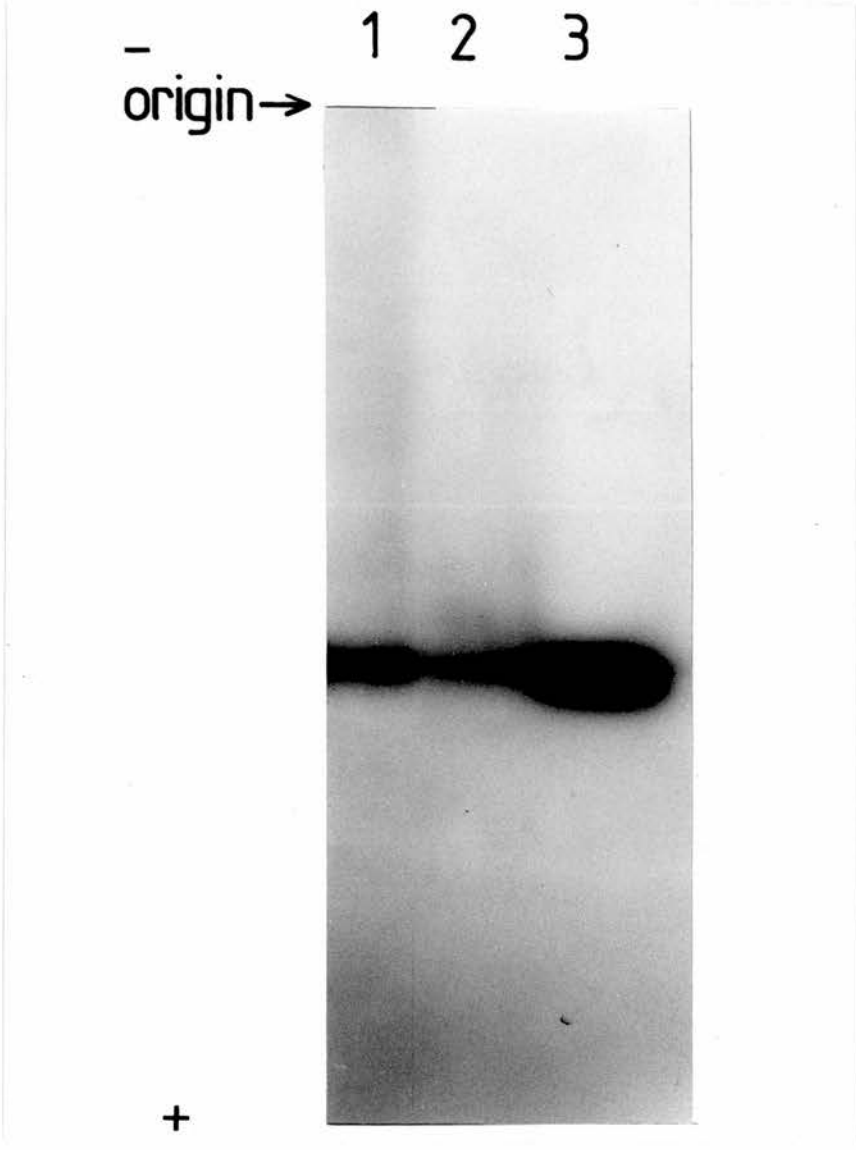


Table 3.8

Estimated M_r of the enzyme fractions in hepatic bile as determined by gradient gel electrophoresis

Fraction	Estimated M_r	
	LAP	ALP
Peak 1	> 1 000 000	> 1 000 000
Peak 3	*	280 000
Peak 4	177 000	180 000

* Not estimable

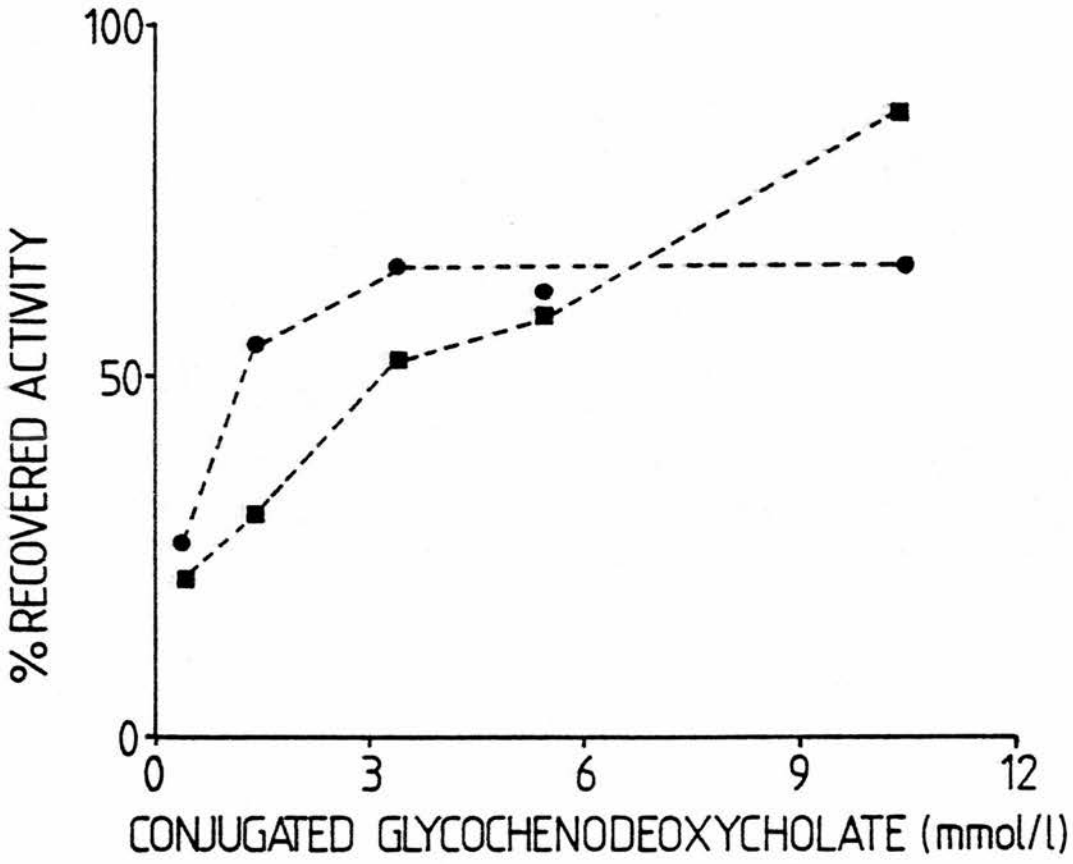
3.10.8 Ultracentrifugation

The results obtained for LAP and ALP were similar to those obtained for γGT (see section 3.8). Over 92% of LAP and ALP activity was recovered from the supernatant following centrifugation at 2500 x g. After further centrifugation of this supernatant at 150 000 x g for 1 h, between 27 and 54% of LAP and 22 and 48% of ALP activity was recovered in the supernatant. Again, when increasing concentrations of glycochenodeoxycholate were added to the bile prior to centrifugation, similar results to those for γGT were obtained, in that more activity of both enzymes was recovered in the supernatant (Fig. 3.20).

3.11 SUMMARY AND DISCUSSION

1. In the absence of detergents, γGT has been shown to be present in human bile largely as a hydrophobic, high M_r form, together with small amounts of a low M_r hydrophilic form of the enzyme.

Figure 3.20 Percentage recovery of enzyme activity in the supernatant following ultracentrifugation of bile at 150 000 x g plotted against conjugated chenodeoxycholate concentration. ● , LAP; ■ , ALP.



2. High $\underline{M}_r$ γGT in human bile is converted both by deoxycholate and by physiological concentrations of glycocholate and glycochenodeoxycholate to a low $\underline{M}_r$ form. This form is hydrophobic and readily reaggregates in the absence of detergents.

3. High $\underline{M}_r$ γGT in human bile is converted, by treatment with the non-ionic detergent Triton X-100, to a molecule of intermediate $\underline{M}_r$.

4. Treatment of human bile with papain converts high $\underline{M}_r$ γGT to a hydrophilic low $\underline{M}_r$ form. This has identical physical properties to the low $\underline{M}_r$ form present in small amounts in native bile and does not reaggregate when added to, and incubated with, bile.

5. When human bile is centrifuged at 150 000 x g, between 25 and 73% of γGT activity is recovered in the supernatant. This proportion is increased following prior addition of bile salts to the bile.

6 Analogous results were obtained for LAP and ALP although the results were incomplete because bile salts inhibited LAP activity and papain destroyed ALP activity.

These results confirm previous reports that γGT, LAP and ALP, when studied by gel chromatography in the absence of detergents, seem to be present in native bile mainly as high $\underline{M}_r$ forms (Price et. al., 1972; Wenham et al., 1978a; Huseby, 1978; Crofton and Smith, 1981a; Echetebu and Moss, 1982a). However, if deoxycholate, Triton X-100 or physiological concentrations of either glycocholate or glycochenodeoxycholate are included in the gel buffer, the elution patterns of the enzymes change markedly. The void volume peak contains less of the enzyme activity and lower $\underline{M}_r$ forms of the enzymes appear (Peak 3, Peak 2T). These lower $\underline{M}_r$ forms reaggregate if they are rechromatographed in detergent-free eluting buffer. The results presented here suggest that, in order to prevent reaggregation from occurring and to obtain a truer picture, gel chromatography must be carried out in buffer containing glycocholate or glycochenodeoxycholate at a concentration of at least 20 mmol/l or 5 mmol/l respectively. The greater efficacy of deoxycholate and glycochenodeoxycholate in causing this change can readily be explained in terms of the greater polarity of glycocholate, due to possession

of an extra hydroxyl group. The results suggest that the apparent high $\underline{M}_r$ of biliary γGT, LAP and ALP may well be an artefact caused by reaggregation of the enzymes when removed into a solution containing no bile salts.

The lower $\underline{M}_r$ forms of γGT, LAP and ALP, which result from bile salt treatment (Peak 3 forms), have been shown to be quite distinct in terms of molecular size, charge and other properties from the low $\underline{M}_r$ forms found in untreated bile or obtained following treatment with papain. The Peak 4 enzymes do not tend to aggregate, show no change of properties on the addition of bile salts and have a significantly smaller $\underline{M}_r$ and negative charge than the Peak 3 enzymes. By analogy with other membrane proteins, the results support previous suggestions (Huseby, 1978) that that Peak 3 forms of γGT (and also LAP and ALP) all contain a hydrophobic domain by which they are normally attached to the hepatocyte or canalicular membrane. They are consistent with explanations that have been advanced previously, namely that bile salt or detergent treatment of γGT complexes results in the formation of a γGT molecule possessing this hydrophobic domain to which detergents may attach (Hughey and Curthoys, 1976). Unless an adequate concentration of detergent is present, enzyme molecules possessing this hydrophobic region will tend to aggregate in aqueous solution. Papain treatment may remove this hydrophobic area, which is presumably a peptide or peptides, causing formation of a γGT molecule which neither binds bile salts, nor reaggregates. It is perhaps not surprising that binding of radiolabelled deoxycholate to the hydrophobic form of γGT was not demonstrated. The approximate molar concentration of γGT in the neat and concentrated samples of bile studied, using the data from Huseby (1977), was estimated to be 7.2 nmol/l and 154 nmol/l respectively. Thus the molar amounts of γGT applied to the column (approximately 15.4 and 308 pmoles respectively) were both several orders of magnitude less than the amount of radiolabelled deoxycholate added to the bile (19.2 nmol) in order to obtain sufficient counts.

The ultracentrifugal studies showed that between 25 and 75% of γGT, LAP and ALP activity in native bile is present in the 150 000 x $\underline{g}$ supernatant. In samples in which the bile salt concentration is low, such as those obtained after several days of biliary drainage, the proportion of each of the enzymes

present in the 150 000 x g supernantant also tended to be low. A similar observation was obtained in vitro by adding glycochenodeoxycholate to bile and thereby increasing the amount of enzyme present in the supernatant fraction. The simple interpretation of these findings is that the 150 000 x g sediment contains particles or aggregated enzyme and that bile salts, whether endogenous or exogenous, will release the enzyme from these complexes. However, the exact nature of these complexes remains to be determined.

Chapter 4

PHYSICAL PROPERTIES OF γ-GLUTAMYLTRANSFERASE IN HUMAN SERUM

γ-Glutamyltransferase in human serum has been shown, by gel chromatography on a variety of media, to be present as three fractions of high, intermediate and low M_r (greater than 600 000, 250 000 - 450 000, and about 100 000 respectively) (Orlowski et al., 1965; Kokot and Kuska, 1968; Huseby, 1978; Wenham et al., 1979; Huseby, 1982a; Huseby, 1982b). Two of these fractions have been studied by several groups of investigators. The high M_r fraction is thought to consist of complexes in which hydrophobic γGT is associated with lipids, lipoprotein-X and other membrane enzymes, or membrane fragments (De Broe et al., 1975; Wenham et al., 1979; Crofton and Smith, 1981b; Huseby, 1982a; Echetebu and Moss, 1982b). In contrast, the low M_r fraction is hydrophilic and is thought to be produced by the action of neutral endopeptidases upon the hydrophobic enzyme either before or after its release into the circulation from the hepatobiliary tract (Huseby, 1978; Tsuji et al., 1980; Huseby, 1982b). However, there has only been one detailed study on the nature or physical properties of the intermediate M_r fraction (Huseby 1982a). This investigation suggested that such intermediate M_r forms consisted of complexes between γGT and high density lipoprotein (HDL).

The present study was undertaken to characterise the γ-glutamyltransferases present in human serum, particularly those of intermediate M_r, and to compare their properties in patients with a wide variety of liver diseases. The objective was two-fold. Firstly, to ascertain if any physical differences between the intermediate M_r forms in different patients might suggest the basis for a useful diagnostic test of liver disease. Secondly, to gain further insight regarding possible mechanisms for the elevation of serum γGT activity in liver disease.

The two enzymes, leucine aminopeptidase and alkaline phosphatase, also thought to originate from the hepatocyte plasma membrane, were included in

the study where appropriate, to see if the findings were applicable on a wider basis to other membrane enzymes.

4.1 SAMPLES USED IN THE STUDY

Sera were obtained from 100 patients with liver disease and 10 apparently healthy individuals in whom there was no clinical or biochemical evidence of hepatic damage. Further details are given in Chapter 5, section 5.1.

4.2 EFFECT OF PAPAIN ON γGT IN HUMAN SERUM

Sera were incubated with papain (1 g/10 g protein) overnight at 20°C in the presence of 100 mmol/l cysteine. The digestion had little effect upon γGT activity (Table 4.1).

Table 4.1

γGT activity after digestion of sera with papain

Sample	% initial activity
Serum A	91
Serum B	107
Serum C	83

4.3 GEL CHROMATOGRAPHY ON SEPHACRYL S300

For the reasons described in section 2.3.4, Sephacryl S300 was chosen, in preference to Sephadex G200, as the gel medium for the study of γGT in human serum. However, in some of the studies on low M_r γGT where Sephacryl offered no additional advantage, Sephadex G200 was used for

convenience, as these studies coincided with equivalent studies on bile described in Chapter 3.

4.3.1 General nomenclature used throughout the chapter

In order to maintain the previous arbitrary nomenclature described in Chapter 3, the peaks have again been numbered in order of their elution from the gel column. Quantitative differences were noted in the relative sizes of the peaks but in this chapter attention is focussed on their physical properties:

Peak 1 (γGT), Peak 1 (LAP), Peak 1 (ALP): void volume for all enzymes.

Peak 2 (γGT), Peak 2 (LAP): major peaks of intermediate $\underline{M}_r$. For γGT, Peak 2 could take two forms (see below). No corresponding peak was present for ALP.

Peak 3 (γGT), Peak 3 (ALP): peaks of low-intermediate $\underline{M}_r$ present in the eluate only when bile salts are present in the eluting buffer. No corresponding peak was present for LAP owing to its inhibition by bile salts.

Peak 4 (γGT), Peak 4 (LAP), Peak 4 (ALP): peaks of low $\underline{M}_r$ representing a small fraction of serum γGT but a major proportion of serum LAP and ALP activity. The size of this peak (for γGT and LAP) was increased greatly in sera that had previously been treated with papain.

4.3.2 Gel chromatography performed in the absence of bile salts

After gel chromatography of normal sera and sera from patients with liver disease, varying amounts of γGT eluted as Peaks 1, 2 and 4 (γGT) (Table 4.2). The recovery of γGT activity was variable; mean 101.7%, range 81 - 146%.

Table 4.2

Distribution of γGT obtained after gel chromatography of 110 sera on Sephacryl S300

Fraction	Percentage of total recovered activity	
	Mean	Range
Peak 1	27.3	6 - 82
Peak 2	57.4	0 - 83
Peak 4	15.3	2 - 58

The elution profile of the second peak of γGT activity from most of the patients with extrahepatic biliary obstruction (Peak 2B (γGT)) differed from that of most of the other patients and normal individuals (Peak 2A (γGT)) (Figs. 4.1, 4.2 and 4.3). Peak 2B (γGT) eluted later than Peak 2A (γGT) and was therefore of lower apparent M_r than Peak 2A (γGT). Of the 25 patients with extrahepatic biliary obstruction who were studied, 20 exhibited Peak 2B (γGT) activity only, three Peak 2A (γGT) only, one approximately equal amounts of both, and one neither peak activity. All sera possessed variable amounts of Peaks 1 and 4 (γGT). It was noted that in some jaundiced sera from patients with pathologies other than extrahepatic biliary obstruction, a shoulder was obtained on Peak 2A (γGT) in the position of Peak 2B (γGT).

4.3.3 Gel chromatography performed in the presence of bile salts

When gel chromatography was performed in the presence of either 12 mmol/l glycochenodeoxycholate or 12 mmol/l deoxycholate, the amount of γGT activity in Peaks 1 and 2 (γGT) decreased, with a corresponding appearance of Peak 3 (γGT) (Fig. 4.4).

Figure 4.1 Elution profile of γGT following gel chromatography on Sephacryl S300 of serum from a healthy individual.

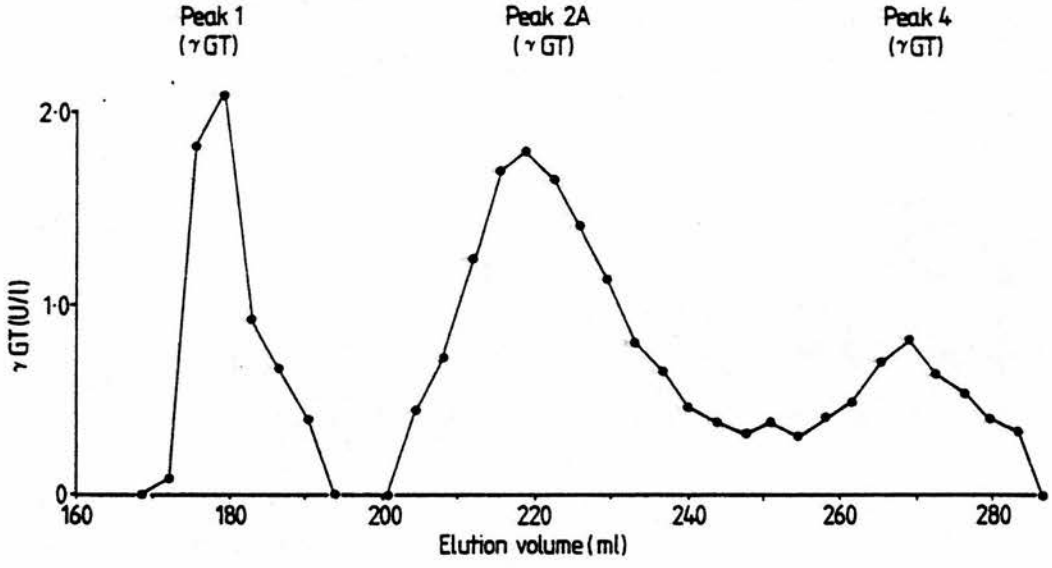


Figure 4.2 Gel chromatography on Sephacryl S300 of γGT in serum from a patient with extrahepatic biliary obstruction.

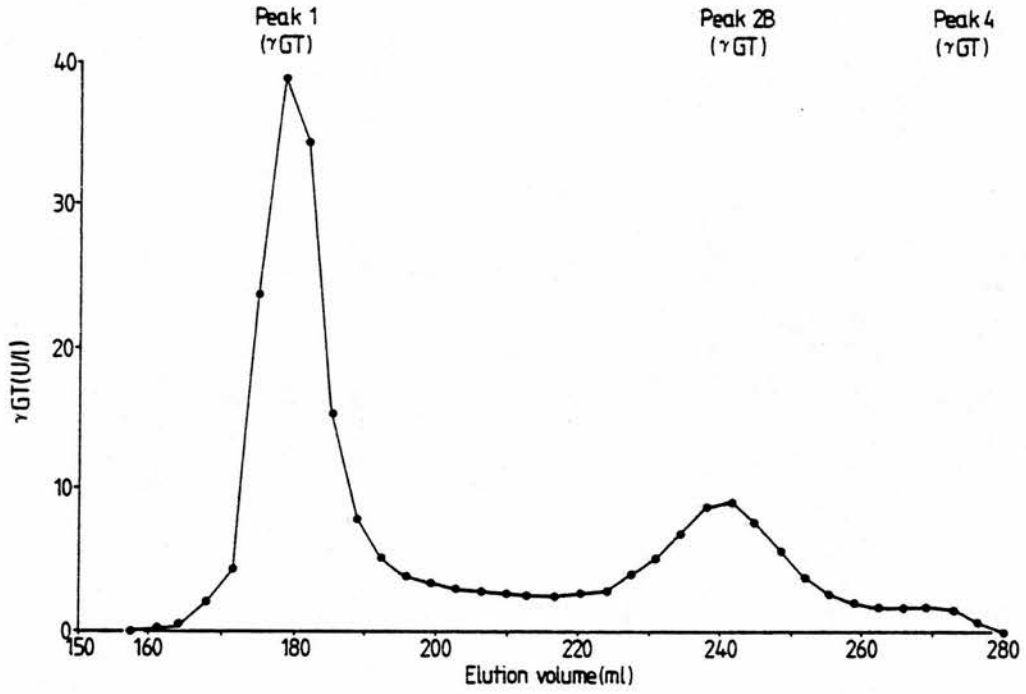


Figure 4.3 Elution profile of γGT following gel chromatography on Sephacryl S300 of serum from a patient with alcoholic cirrhosis.

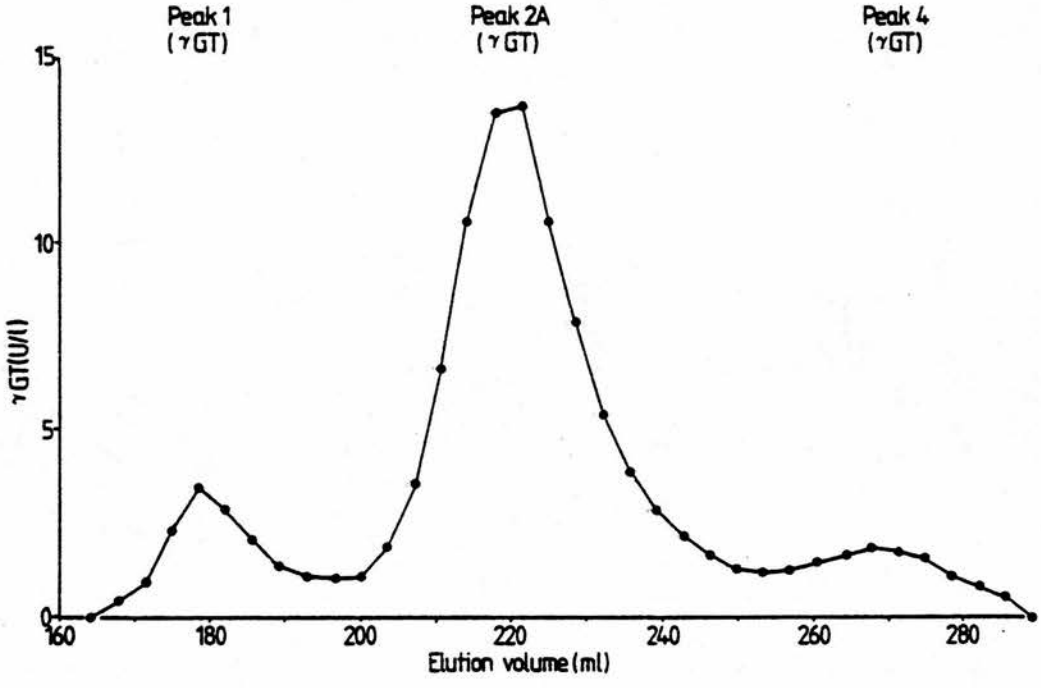
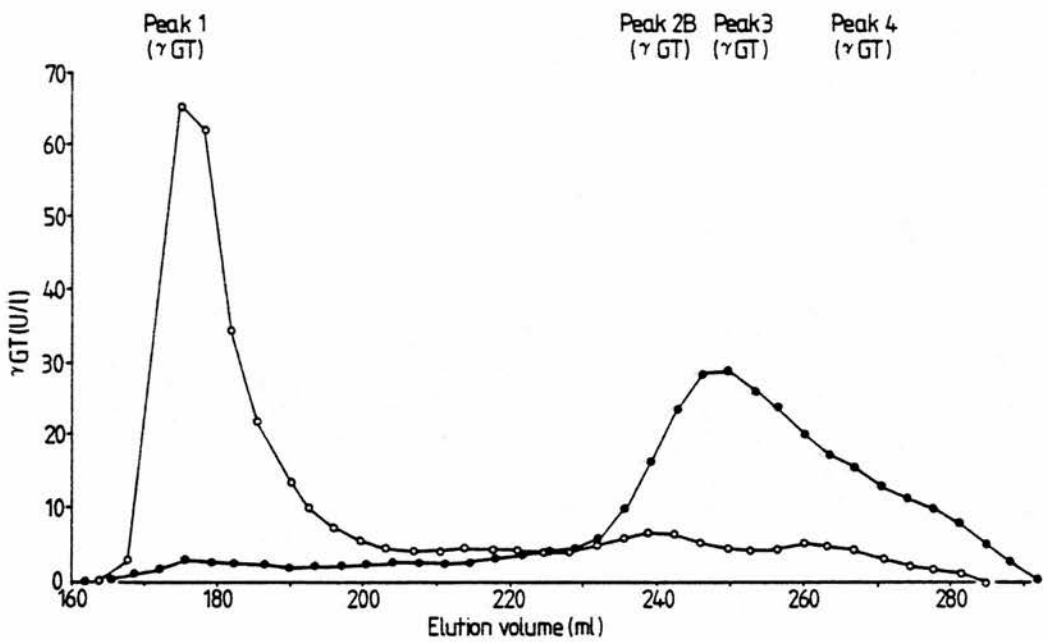


Figure 4.4 Gel chromatography on Sephacryl S300 of serum from a patient with extrahepatic biliary obstruction, with and without 12 mmol/l deoxycholate in the elution buffer. ○ , no deoxycholate ● , 12 mmol/l deoxycholate.



The fractions containing Peaks 1 and 2 (γGT) obtained from gel chromatography were pooled, concentrated by ultrafiltration and rechromatographed in the presence of either 12 mmol/l glycochenodeoxycholate or 12 mmol/l deoxycholate. Following rechromatography, Peak 1 (γGT) and Peak 2 (γGT) disappeared, giving rise to Peak 3 (γGT) (Fig. 4.5).

4.3.4 Gel chromatography of papain-treated sera

Following gel chromatography of papain-treated sera in the absence of bile salts, there was a decrease in γGT activity eluting as Peaks 1 and 2 (γGT), together with an increase in activity eluting as Peak 4 (γGT) (Fig. 4.6).

The estimated M_r values of all of the various fractions obtained after gel chromatography are shown in Table 4.3.

Table 4.3

Estimated M_r values of the γGT fractions obtained after gel chromatography on Sephacryl S300

Fraction	Estimated M_r
Peak 1	>1 000 000
Peak 2A	325 000 - 500 000
Peak 2B	258 000 - 295 000
Peak 3	175 000
Peak 4	118 000

Figure 4.5 Gel chromatography on Sephacryl S300 of Peak 2B (γGT) with ● , and without ○ , 12 mmol/l deoxycholate in the elution buffer.

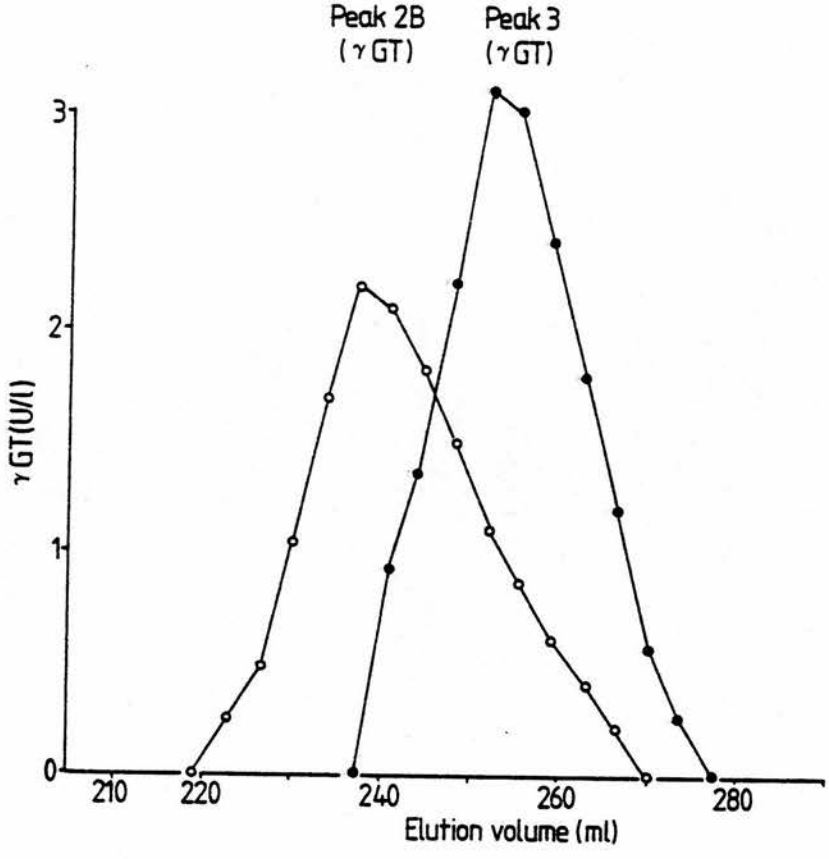
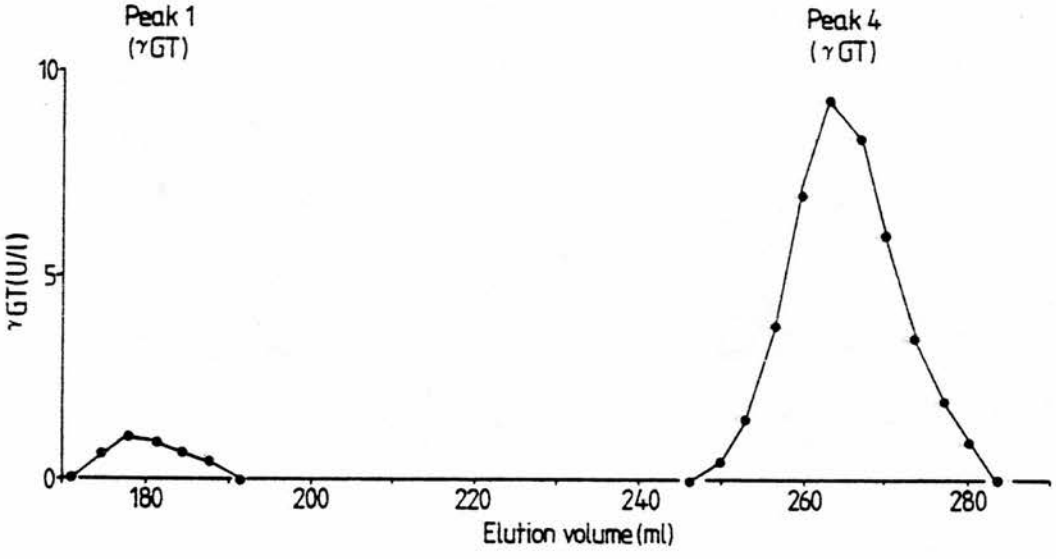


Figure 4.6 Elution profile on Sephacryl S300 of papain-treated serum from a patient with liver disease.



4.4 7% POLYACRYLAMIDE GEL ELECTROPHORESIS

4.4.1 Electrophoresis in the absence of bile salts

Two fairly distinct patterns of γGT activity, according to the type of liver disease, were obtained following electrophoresis on 7% polyacrylamide gel. In sera from patients with extrahepatic biliary obstruction, two main bands of activity were seen, one at the origin (Band 1 (γGT)), and another of a mobility 45 - 55% of that of albumin (Band IIB (γGT)). Occasionally, smaller, minor zones of slower mobility were seen. The sera from other groups of patients and the normal sera showed a smaller zone of activity at the origin, with significant activity occurring in various zones (Bands IIA (γGT)), with mobilities between 8 and 40% of that of albumin. Minor zones were often present, corresponding to Band IIB (γGT), in abnormal but not in normal sera. Sera from all patients showed a less intense zone of activity (Band IV (γGT)), of mobility between 70 and 80% of that of albumin. This zone was also present in normal sera but seemed to contribute a greater proportion of the total activity. Typical electrophoretic patterns are shown in Fig. 4.7. The positions of the various bands are shown schematically in Fig. 4.8.

Electrophoresis was performed on concentrated pooled fractions of Peak 1 (γGT), Peak 2A (γGT), Peak 2B (γGT) and Peak 4 (γGT) obtained after gel chromatography. This showed that Peak 2A (γGT) was heterogenous, giving rise to several zones (Bands IIA (γGT)). On the other hand, Peak 1 (γGT), Peak 2B (γGT) and Peak 4 (γGT) each gave rise to an electrophoretically discrete band (Band I (γGT)), Band IIB (γGT) and Band IV (γGT) respectively) (Fig. 4.9).

4.4.2 Electrophoresis performed in the presence of bile salts

Electrophoresis, in the presence of a concentration of sodium deoxycholate greater than or equal to 5 mmol/l, gave rise to two bands of activity. The faster one (Band III (γGT)), was the main band obtained after electrophoresis of either serum, or of a concentrated pool of Peak 3 (γGT) obtained after gel chromatography. The other band (Band IV (γGT)) was the main band obtained following electrophoresis of a concentrated pool of Peak 4 (γGT) (Fig. 4.10).

Figure 4.7 7% polyacrylamide gel electrophoresis of γGT in sera from patients with liver disease. 1, extrahepatic biliary obstruction; 2, blank; 3-7, alcoholic cirrhosis.

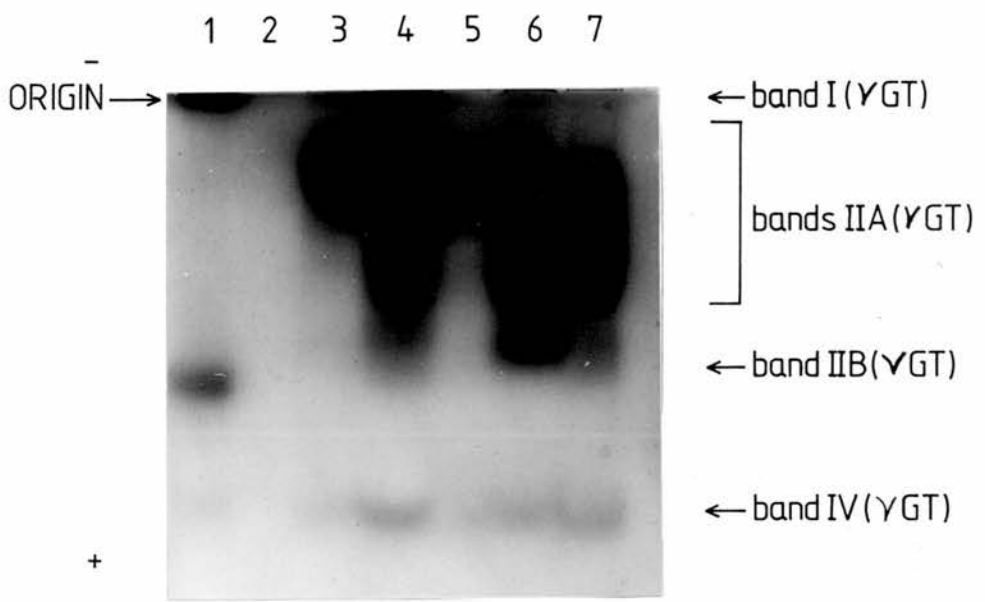
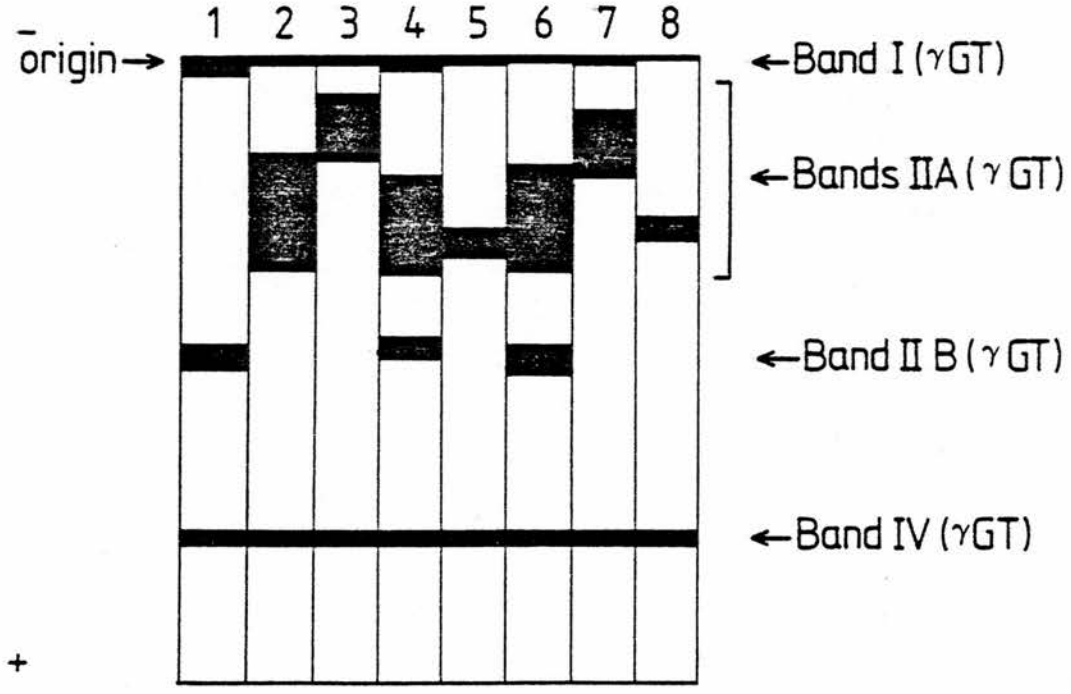


Figure 4.8 Schematic representation of the positions of the various zones of γGT activity obtained after electrophoresis of sera in polyacrylamide gel. 1 - 7, sera from patients with 1, extrahepatic biliary obstruction; 2, alcoholic cirrhosis; 3, chronic active hepatitis; 4, primary biliary cirrhosis; 5, haemochromatosis; 6, alcoholic cirrhosis; 7, liver metastases; 8, normal serum.



e 4.9 Polyacrylamide gel electrophoresis of serum and the fractions obtained after gel chromatography, stained for γGT activity. 1, Peak 1 (γGT); 2, Peak 2B (γGT); 3, Peak 2A (γGT); 4, Peak 4 (γGT); 5, serum. Track 4 contains slight contamination in the position of Band IIA (γGT) due to overflow of staining from an adjacent track. Unfortunately, the zone corresponding to Band IV (γGT) in track 4 did not show up very clearly after photography.

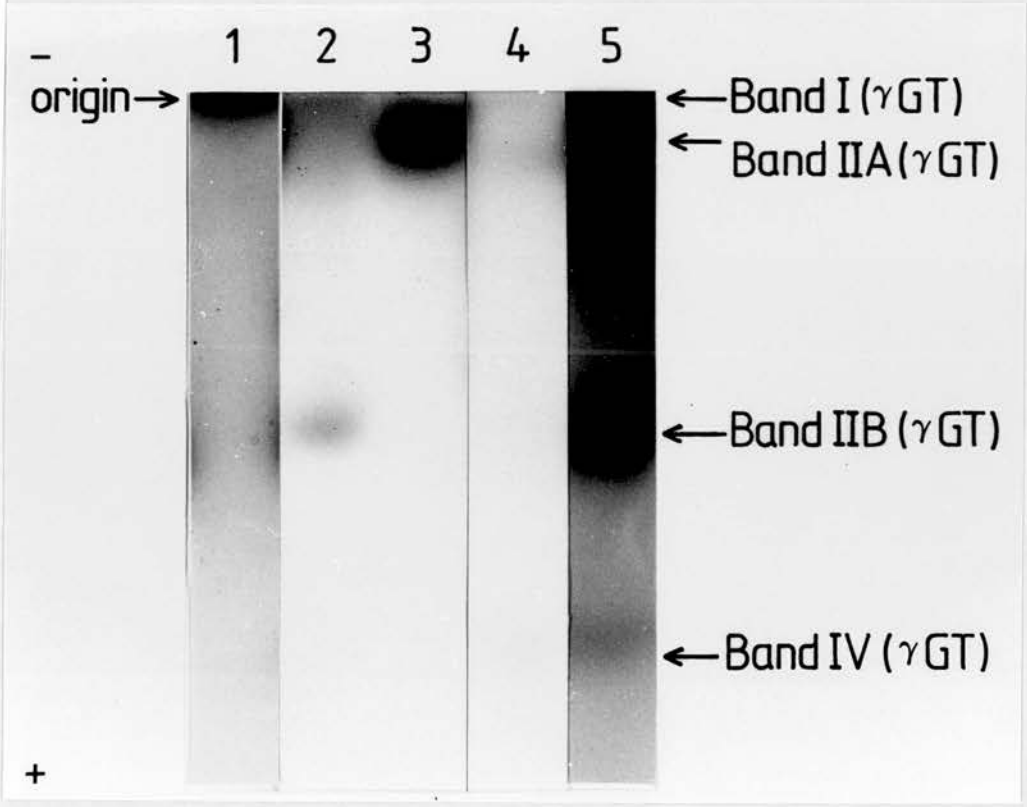
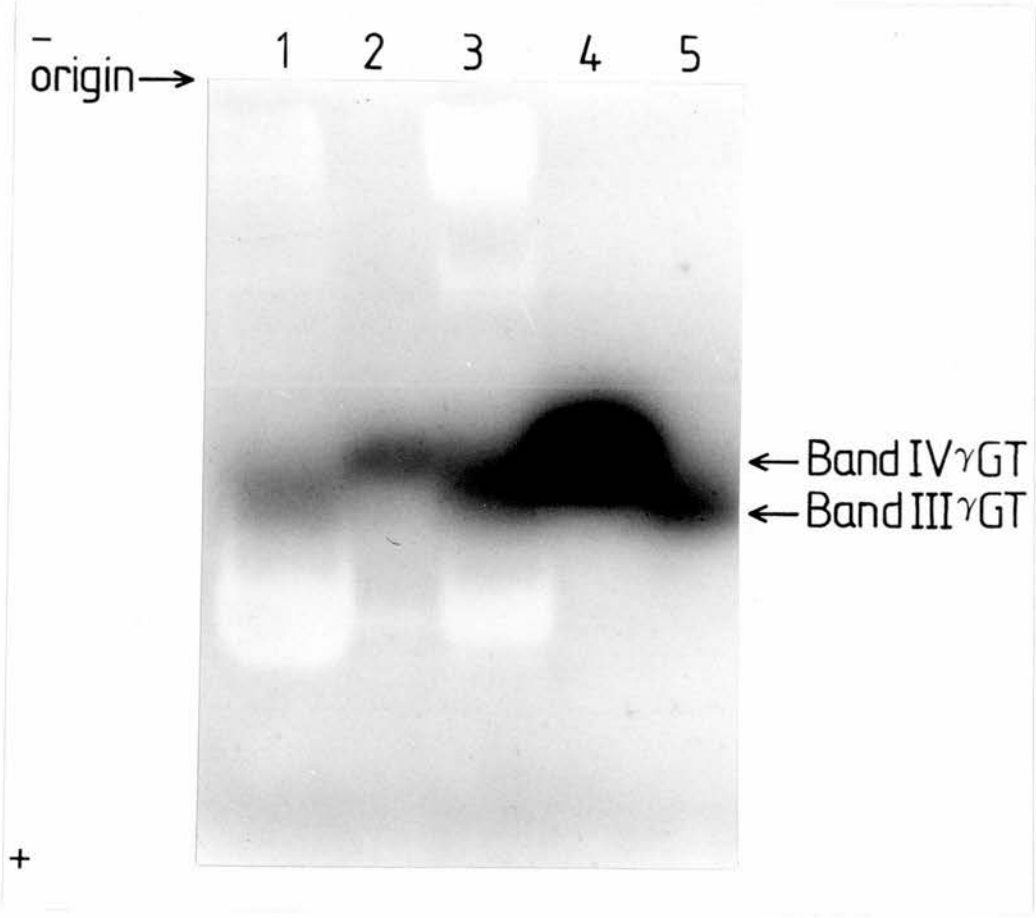


Figure 4.10 Electrophoresis on 7% polyacrylamide gel of sera and the fractions obtained after performing gel chromatography. Both the gel and running buffer were equilibrated with 5 mmol/l sodium deoxycholate. 1, 3, sera; 2, 4, Peak 4 (γGT); 5, Peak 3 (γGT).



4.5 POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS

Electrophoresis, carried out in the absence of bile salts demonstrated the heterogeneity of Peak 2 (γGT). In the presence of 12 mmol/l deoxycholate, the technique confirmed that Peak 3 (γGT) was of higher M_r than Peak 4 (γGT) (Table 4.4).

Table 4.4

Estimated M_r values of the various γGT fractions

Fraction	Estimated M_r
Peak 1	>1 000 000
Peak 2A	300 000 - 710 000
Peak 2B	180 000 - 220 000
Peak 3	135 000
Peak 4	98 000

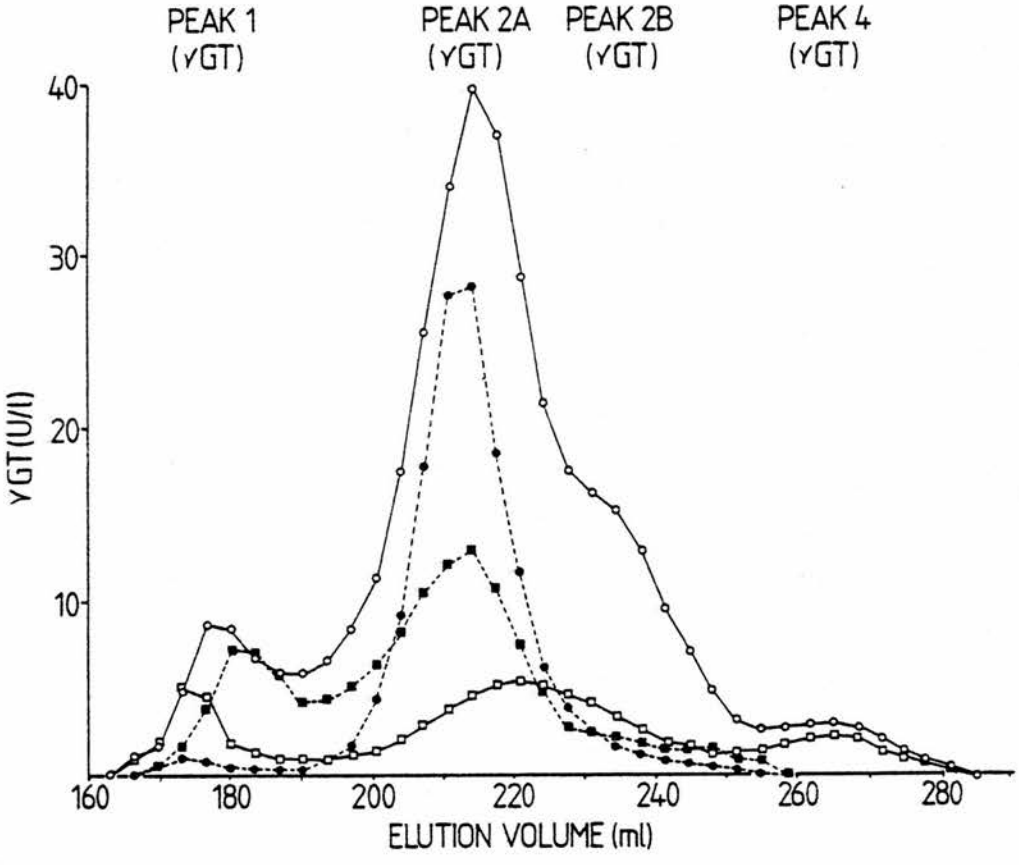
4.6 POLYANION PRECIPITATION

This was performed on 14 sera, as described in section 2.5.2, using dextran sulphate and manganese chloride. Gel chromatography was then performed on the redissolved lipoprotein fractions of LDL plus VLDL, and HDL.

Between 40 and 75% of Peak 1 (γGT) activity co-precipitated with the LDL and VLDL fraction, together with a much smaller proportion of Peak 2A (γGT) (Fig. 4.11). Between 49 and 65% of Peak 2A (γGT) was precipitated with the HDL fraction, whereas Peak 2B (γGT) did not co-precipitate with any of the lipoprotein fractions.

Polyanion precipitation of the lipoprotein fractions was also carried out on 2 sera using sodium phosphotungstate and magnesium chloride as described in section 2.5.1. Gel chromatography was performed on the redissolved

Figure 4.11 Elution profile of γGT in serum and the lipoprotein fractions from a patient with alcoholic liver disease before and after precipitation with polyanions. ○ , whole serum; ■ , LDL, VLDL; ● , HDL; □ , supernatant after precipitation of LDL, VLDL and HDL.



lipoprotein fractions. Similar results were obtained to those using dextran sulphate and manganese chloride, in that Peak 1 (γGT) co-precipitated predominantly with LDL plus VLDL, whereas Peak 2A (γGT) mostly co-precipitated with HDL.

The results described here for Peak 2A (γGT) provide circumstantial evidence that this fraction is a part of HDL. However, in case the polyanion precipitation procedure coincidently precipitates γGT protein, the following control experiments were performed.

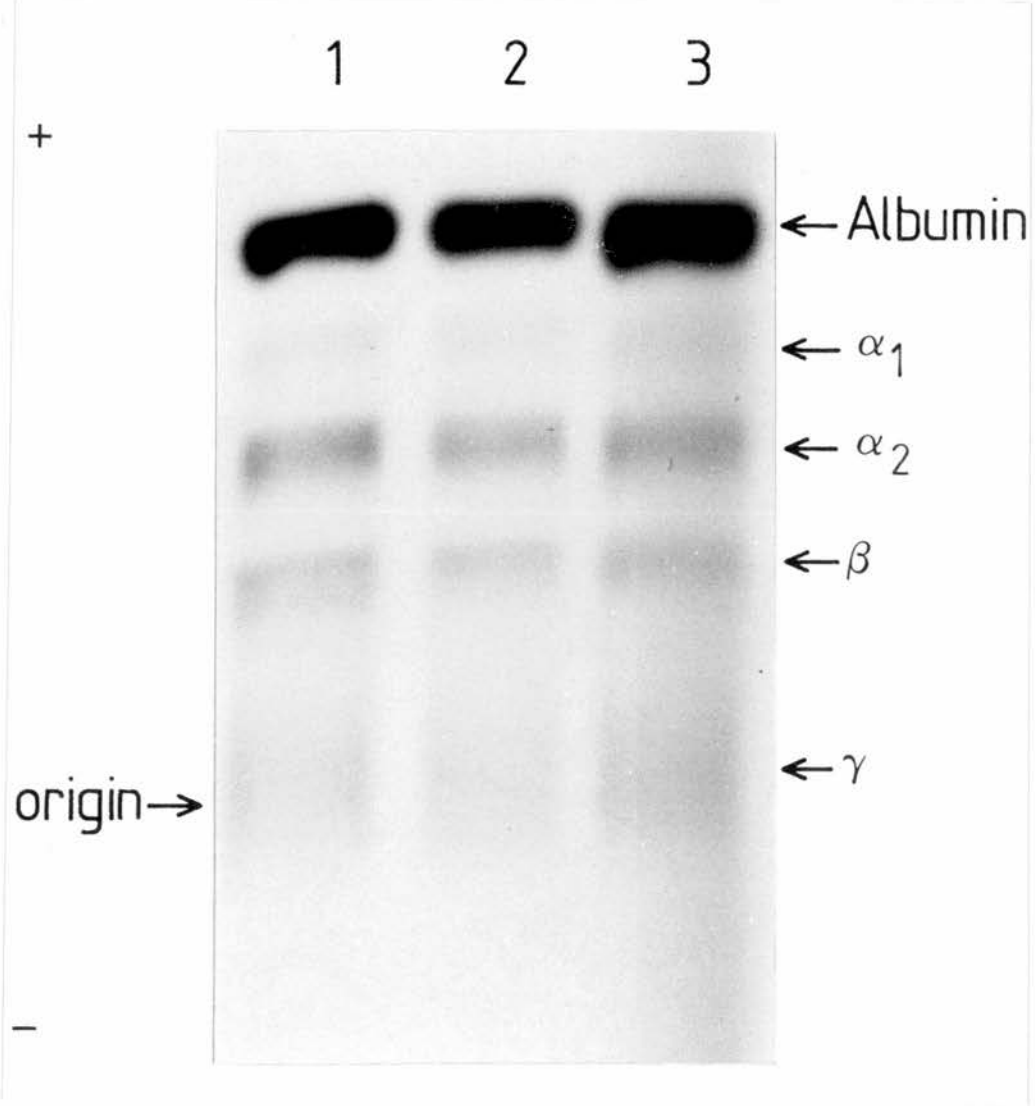
Firstly, the polyanion procedure using dextran sulphate and manganese chloride was performed on a serum sample and the total protein determined in the supernatants after each precipitation stage. Electrophoresis on cellulose acetate, followed by protein staining, was performed on each of the supernatants and on the original serum. The results are shown in Fig. 4.12 and Table 4.5 and suggest that the polyanion procedure has very little effect on the total recovered protein from either the supernatant or the various protein fractions.

Table 4.5

Total recovered protein in the supernatant following precipitation of the various lipoprotein fractions

Fraction	Total protein (g/l)	Percentage initial concentration
Untreated serum	69	100
Serum minus LDL and VLDL	69	100
Serum minus LDL, VLDL and HDL	65	94

Figure 4.12 Cellulose acetate electrophoresis of serum before and after polyanion precipitation of the various lipoprotein fractions, stained for protein. 1, whole serum; 2, serum minus LDL and VLDL; 3, serum minus LDL, VLDL and HDL.



Secondly, Peak 4 (γGT) was added to serum (from which HDL and LDL plus VLDL had been removed) at a concentration of dextran sulphate and manganese chloride suitable for HDL precipitation. The mixture was centrifuged for 30 min at 20 000 x g and the γGT activity determined on the supernatant. 91% of the added γGT activity was recovered in the supernatant, suggesting that the polyanion procedure did not precipitate significant quantities of γGT protein.

4.7 INCUBATION OF SERA WITH ANTISERUM TO APOLIPOPROTEIN A

4.7.1 Optimisation of antiserum/serum ratio

10 µl of serum was incubated with increasing amounts of antiserum to apolipoprotein A or with saline as a control (0 - 50 µl) overnight at room temperature. The volumes of all of the incubations were made up to 60 µl with saline. For each serum-apolipoprotein antiserum incubation there was an equivalent saline-apolipoprotein antiserum incubation of the same volume ratio in order to correct for γGT activity in the antiserum. The mixture was centrifuged at 11 000 x g for 10 min and γGT activity determined on the supernatant. The percentage of γGT activity precipitated in each incubation was calculated according to the equation:

$$\% \gamma GT \text{ activity precipitated} = \left(1 - \frac{(A - C)}{B}\right) \times 100$$

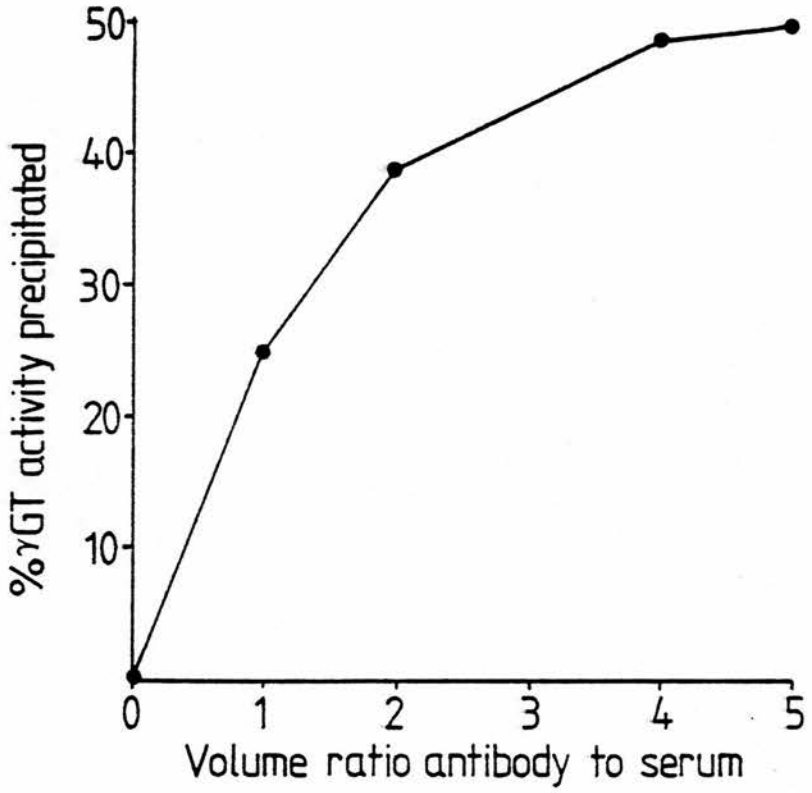
Where A = γGT activity in the supernatant of the serum/apolipoprotein antiserum mixture.

B = γGT activity in the corresponding serum/saline control mixture.

C = γGT activity in the corresponding saline/apolipoprotein control mixture.

The results are shown in Fig. 4.13 and suggest that a volume ratio of antiserum to serum of 5 to 1 gives optimal precipitation of γGT activity.

Figure 4.13 Percentage of γGT activity precipitated following incubation of serum with increasing amounts of antiserum to apolipoprotein A.



4.7.2 Effect of ammonium sulphate on the precipitation of the antibody/γGT/HDL complex

This brief investigation was carried out to maximise the precipitation of the antibody/γGT/HDL complex. 10 µl of another serum was incubated with 50 µl of antiserum to apolipoprotein A and with 50 µl of saline as a control overnight at room temperature. 10 µl of saline was also similarly incubated with 50 µl of antiserum as an antiserum control. Increasing amounts of ammonium sulphate (270 g/l) were then added, mixed, and the mixture centrifuged for 10 min at 11 000 x g. The results are shown in Fig. 4.14 and show that maximal precipitation is obtained after the addition of 1 ml of ammonium sulphate. Ammonium sulphate did not precipitate γGT activity in the absence of antibody.

4.7.3 Quantitative investigation into the precipitation of serum γGT activity with antiserum to apolipoprotein A

The following incubation tubes were set up:

A 50 µl of antiserum to apolipoprotein A + 10 µl of serum (69 patients) or 10 µl of concentrated pool of Peak 4 (γGT).

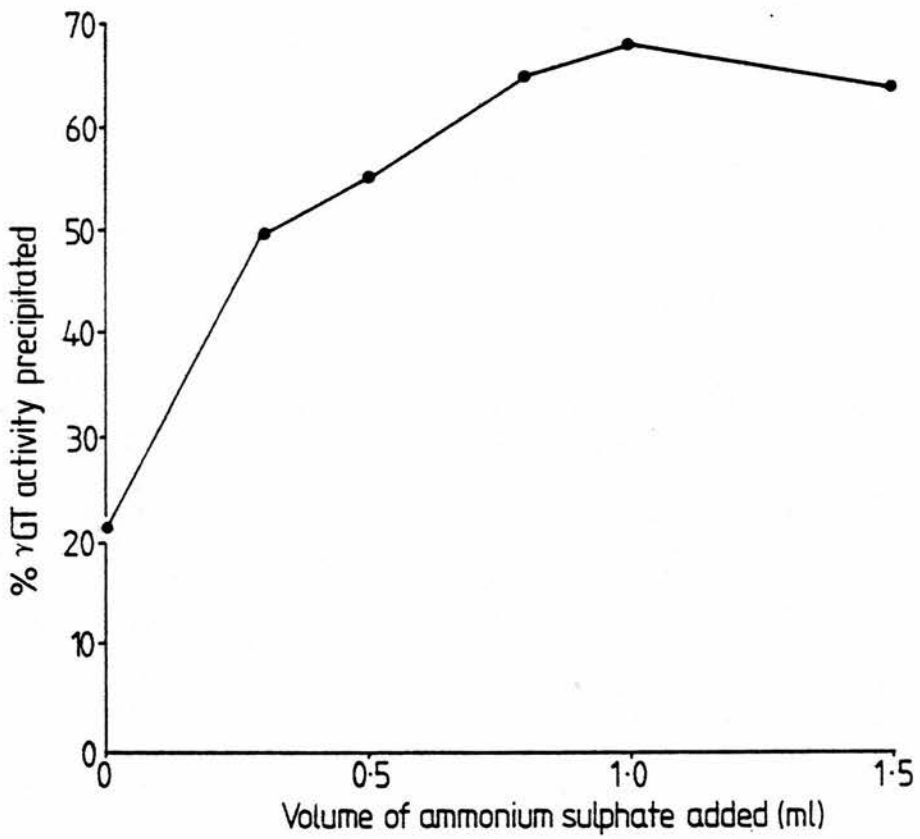
B As above, but with saline substituted for antiserum to apolipoprotein A.

C 50 µl of antiserum to apolipoprotein A + 10 µl of saline as a batch control to correct for the γGT activity of the antibody.

These were incubated overnight at room temperature, 1 ml of ammonium sulphate was added, mixed, and centrifuged for 10 min at 11 000 x g. The percentage of γGT activity precipitated in each incubation was calculated as described in section 4.7.1.

Variable amounts of γGT activity were precipitated after incubation of sera with antiserum to apolipoprotein A (mean 59.5%; range 8 - 92%). In contrast,

Figure 4.14 Effect of increasing the amount of ammonium sulphate upon the percentage of γGT activity precipitated after incubation of serum with antiserum to apolipoprotein A.



no Peak 4 (γGT) activity was precipitated by the incubation procedure. Similarly, incubation of 11 sera with antiserum to apolipoprotein B (raised in the same animal species as the antiserum to apolipoprotein A and using the same incubation conditions as above) did not cause precipitation of any serum γGT activity.

4.7.4 Qualitative investigation into binding of serum γGT to antiserum to apolipoprotein A

25 µl of serum, papain-treated serum, or concentrated Peak 4 (γGT) pool were incubated overnight at room temperature with 125 µl of antiserum to apolipoprotein A or with saline as a control. 25 µl of 40% (w/v) sucrose solution containing 0.1% (w/v) bromophenol blue were added to label the albumin and 150 µl of the mixture were subjected to electrophoresis on either 7% polyacrylamide or 4 -30% polyacrylamide gradient gels. The gels were stained for γGT activity.

Incubation of serum with antiserum to apolipoprotein A resulted in a marked decrease in staining associated with Bands IIA (γGT) and Band IIB (γGT) when compared with incubation of serum with a saline control (Fig. 4.15). Inspection of the gels also suggested an increase in intensity of staining of γGT at the origin.

On the other hand, incubation of the papain-treated sera or Peak 4 (γGT) with antiserum to apolipoprotein A did not alter the staining of Band IV (γGT) when compared with incubation with a saline control (Fig. 4.16).

Immunoelectrophoresis against antiserum to apolipoprotein A was performed on 4 patients' sera and the precipitin arcs stained for γGT activity. The results (Fig. 4.17) show that the precipitin arcs did stain for γGT activity, although there was significant residual background activity due to non-precipitated enzyme, despite the two day washing procedure. In the absence of substrate, no staining of the precipitin arcs was observed.

Figure 4.15 Polyacrylamide slab gel electrophoresis of sera from patients with liver disease. 1, 3, 5, 7, 9, after incubation with saline; 2, 4, 6, 8, 10, after incubation with antiserum to apolipoprotein A; 11, antiserum to apolipoprotein A.

Figure 4.16 Polyacrylamide slab gel electrophoresis of papain-treated serum and Peak 4 (γGT). Papain-treated serum 2, after incubation with saline, 1, after incubation with antiserum to apolipoprotein A; Peak 4 (γGT) 4, after incubation with saline; 3, after inaubation with antiserum to apolipoprotein A.

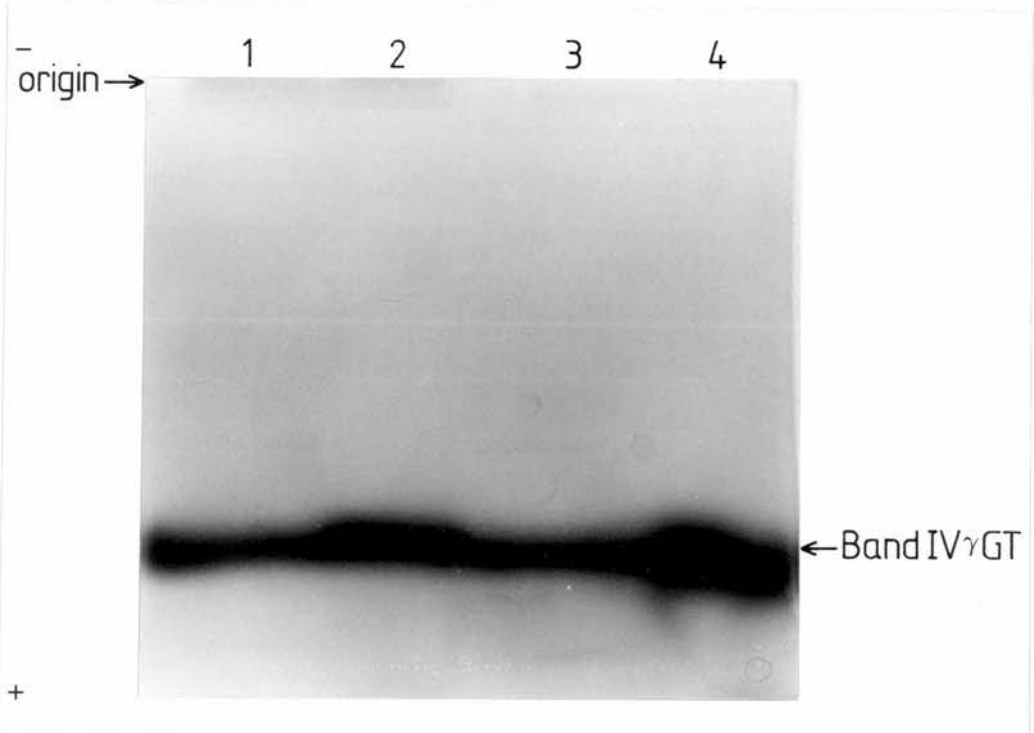
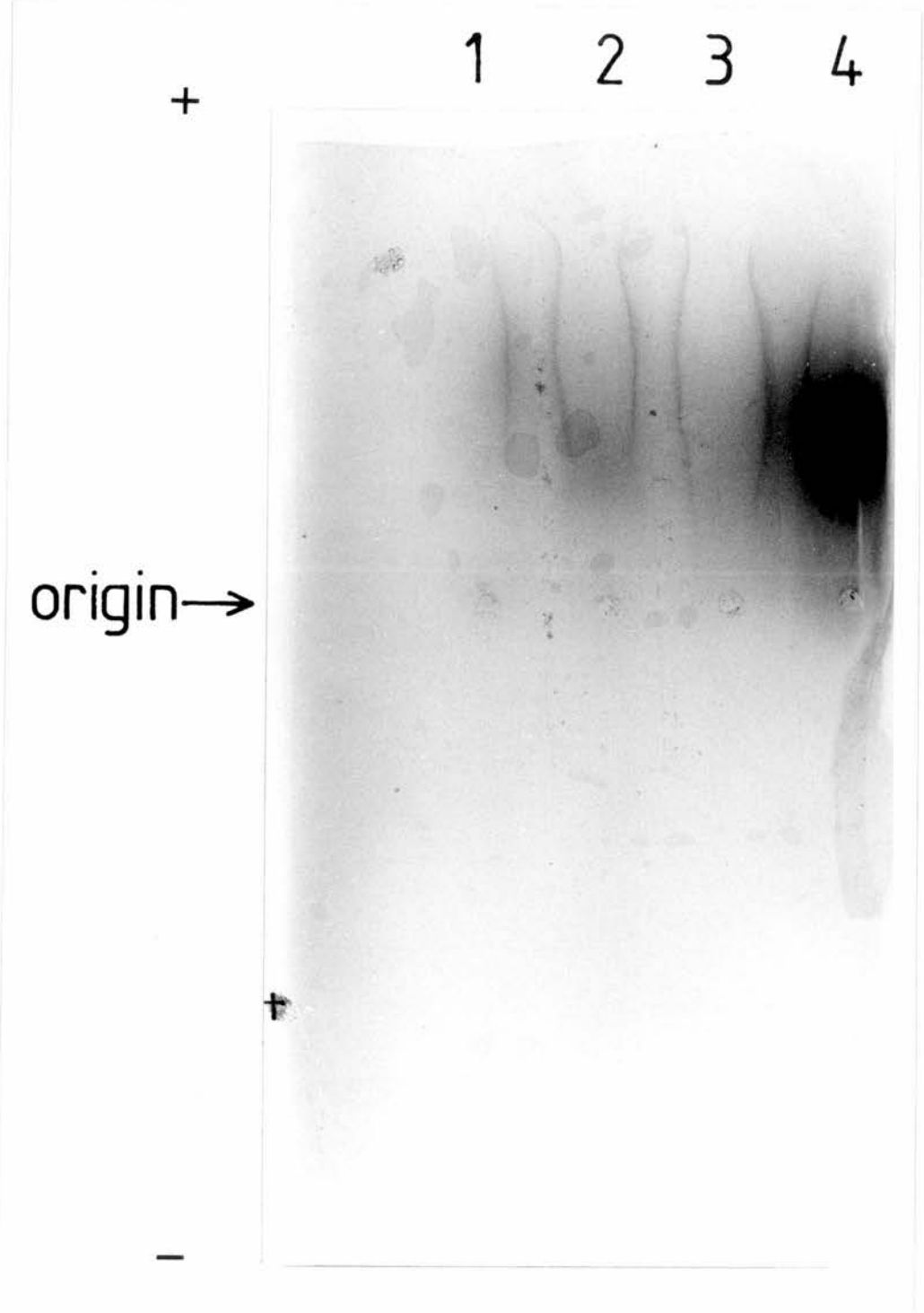


Figure 4.17 Immunoelectrophoresis of 4 sera against antiserum to apolipoprotein A. The precipitation arcs are stained for γGT activity. 1, 2, sera from patients post liver transplant; 3, 4, sera from patients with alcoholic cirrhosis.



As a means of 1) validating the antiserum and 2) showing that the enzyme staining of the precipitin arcs described above was not due to any inherent γGT activity associated with the antiserum, immunoelectrophoresis against antiserum to apolipoprotein A was performed on the various fractions obtained after polyanion precipitation. Immunoelectrophoresis of the LDL plus VLDL fraction, and of the HDL-free serum did not reveal the presence of any precipitin arcs when stained for protein. On the other hand, immunoelectrophoresis of the HDL fraction gave rise to a single precipitin arc in the α_1-globulin region when stained for protein (Fig. 4.18). No enzyme activity was observed when this precipitin arc was stained for such activity.

The results of this section provide additional evidence that the γGT fractions of intermediate $\underline{M}_r$ are a part of HDL.

4.8 INCUBATION OF SERA WITH BILE

These investigations were carried out in an attempt to reproduce the serum abnormalities found in extrahepatic biliary obstruction.

Sera containing Peak 2A (γGT) activity were incubated with an equal volume of hepatic bile for 3 h at 37°C. The mixture was then subjected to gel chromatography on Sephacryl S300, or electrophoresis on either 7% polyacrylamide or 4 - 30% polyacrylamide gradient gels.

Gel chromatography of the mixture revealed that the Peak 2A (γGT) activity appeared to change to Peak 2B (γGT) activity (Fig. 4.19). This change was occasionally associated with an apparent increase in total γGT activity recovered from the sera/bile mixtures. This increase may be due to a matrix effect of the serum proteins upon biliary γGT.

After electrophoresis of the mixture, there was a marked decrease in the intensity of Bands IIA (γGT) along with the appearance of a zone (Band IIC (γGT)) ahead of Band IIB (γGT) (Fig. 4.20). Similarly, electrophoresis of bile-treated sera from patients with obstructive jaundice possessing Band IIB (γGT) activity, resulted in the appearance of Band IIC (γGT). Usually this was associated with a decrease in the intensity of staining of Band IIB (γGT).

Figure 4.18 Immunoelectrophoresis against antiserum to apolipoprotein A of whole serum and the lipoprotein fractions obtained after the polyanion precipitation procedure. 1, whole serum; 2, supernatant after precipitation of LDL and VLDL; 3, supernatant after precipitation of LDL, VLDL and HDL; 4, LDL and VLDL; 5, HDL; 6, Blank; 7, serum protein fractions.

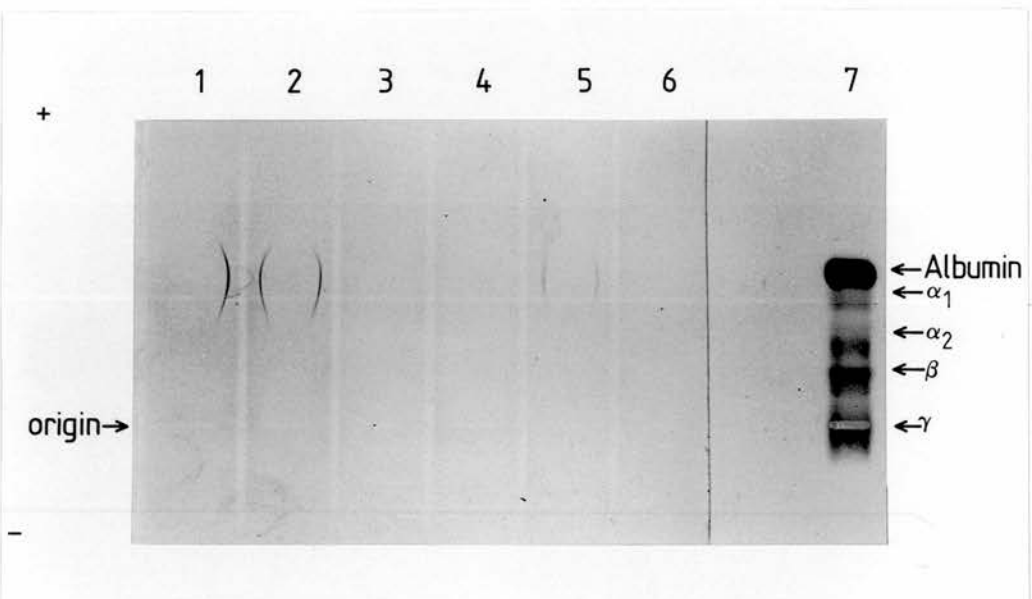


Figure 4.19 Elution profile on Sephacryl S300 of γGT in the serum of a patient with alcoholic cirrhosis before and after incubation with hepatic bile. ○ , serum before; ■ , serum and bile mixture; ● , hepatic bile.

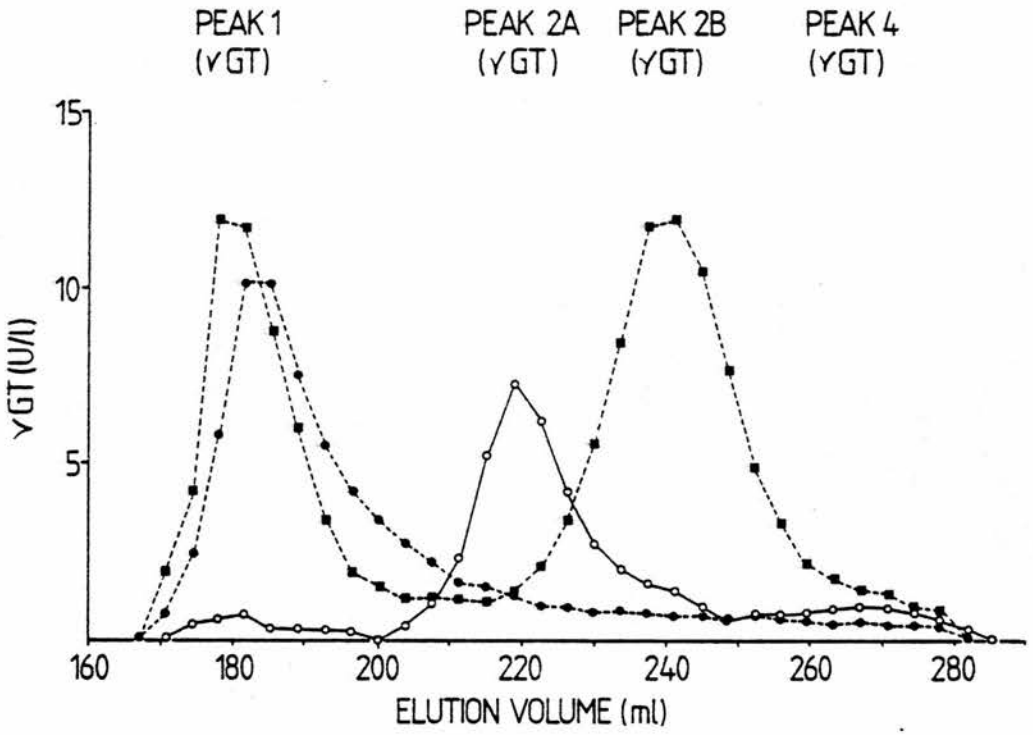
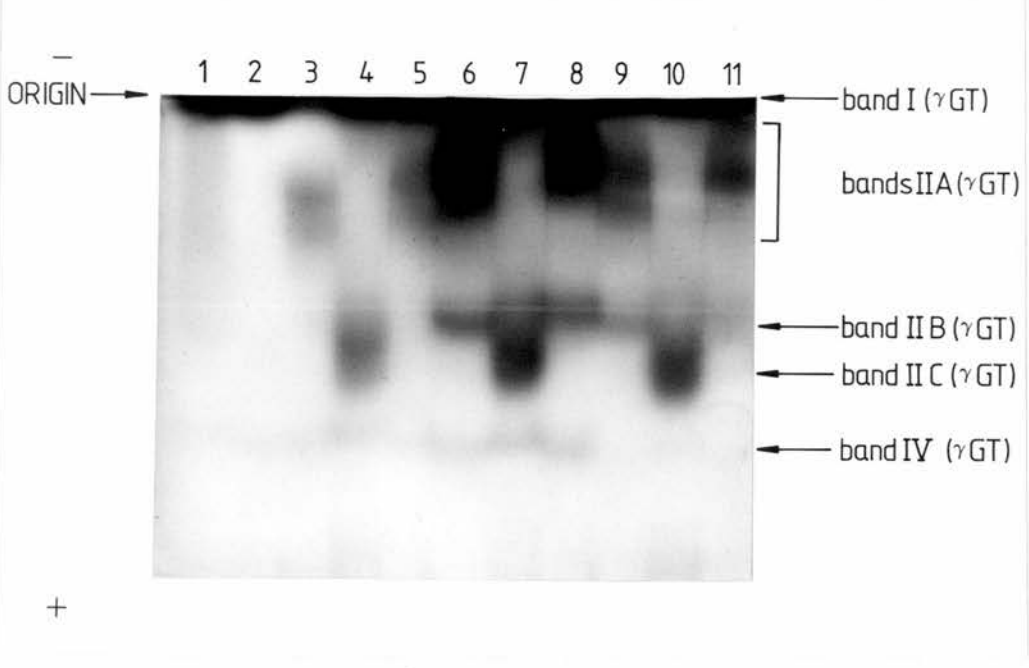


Figure 4.20 Polyacrylamide slab gel electrophoresis of sera from patients with liver disease before and after incubation with dialysed or undialysed hepatic bile. 1, undialysed hepatic bile; 2, dialysed hepatic bile; 3, 6, 9, sera + saline; 4, 7, 10, sera + undialysed bile; 5, 8, 11, sera + dialysed bile.



The ability of bile to effect these changes of Peak 2A (γGT) and Bands IIA (γGT) and IIB (γGT) was inhibited by prior dialysis of the bile against 1000 volumes of 20 mmol/l Tris-HCl buffer, pH 8.0, overnight at 4°C, and for a further 12 h at 4°C after a change of buffer (Figs. 4.20 and 4.21).

Incubation of the various bile-sera mixtures with antiserum to apolipoprotein A, followed by electrophoresis, resulted in a decrease in the intensity of staining of Band IIC (γGT) when compared to mixtures that had been incubated with a saline control (Fig. 4.22).

4.9 INCUBATION OF SERA WITH BILE SALTS

The results of section 4.8 suggest that a small dialysable molecule present in bile was responsible for effecting the changes on Peaks 2A (γGT) and Bands IIA (γGT) and IIB (γGT). Since bile salts were the most obvious candidates for this role, the effect of bile salts on serum was investigated further.

Patients' sera were incubated with an equal volume of glycochenodeoxycholate at concentrations ranging from 10 μmol/l to 20 mmol/l for 3 h at 37°C. Electrophoresis of the mixtures on either 7% polyacrylamide or 4 - 30% polyacrylamide gradient gels showed that incubation with glycochenodeoxycholate at a concentration greater than or equal to 6 mmol/l resulted in a decrease in intensity of Bands IIA (γGT) and IIB (γGT) together with the appearance of Band IIC (γGT) (Fig. 4.23). This phenomenon appeared to be independent of the initial concentration of total conjugated chenodeoxycholate in the sera studied. For the patients with extrahepatic biliary obstruction this was: mean, 64 μmol/l; range, 9 - 229 μmol/l. For the others the mean was 12 μmol/l, range 0 - 113 μmol/l.

A sample of serum, from an alcoholic patient, containing Peak 2A (γGT) activity was incubated with an equal volume of 20 mmol/l glycochenodeoxycholate for 3 h at 37°C. Gel chromatography of the mixture on Sephacryl S300 revealed a shift in the elution profile of Peak 2A (γGT) to that approximating to Peak 2B (γGT). No change in the elution profile of Peak 1 (γGT) was observed (Fig. 4.24).

Figure 4.21 Elution profile on Sephacryl S300 of γGT in the serum of a patient with alcoholic cirrhosis before and after incubation with dialysed hepatic bile. ○ , serum before; ● , serum and bile mixture.

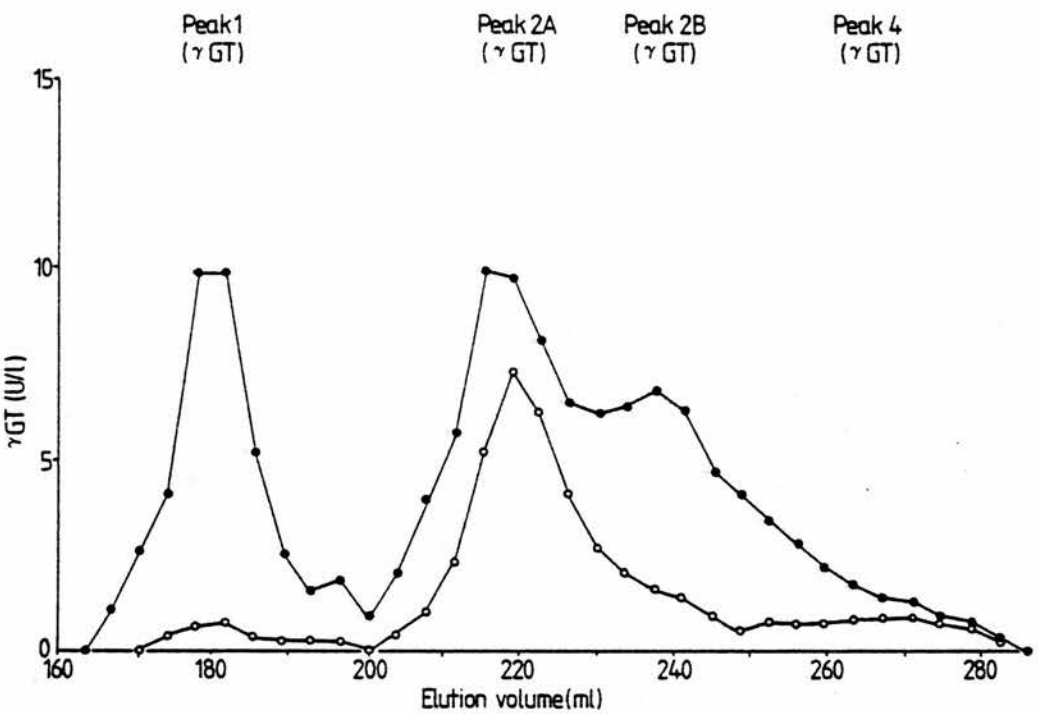


Figure 4.22 Polyacrylamide gradient gel electrophoresis of various bile-sera mixtures before and after incubation with antiserum to apolipoprotein A. 1, serum before; 2, serum + bile before; 3, serum after; 4, serum + bile after; 5, bile after.

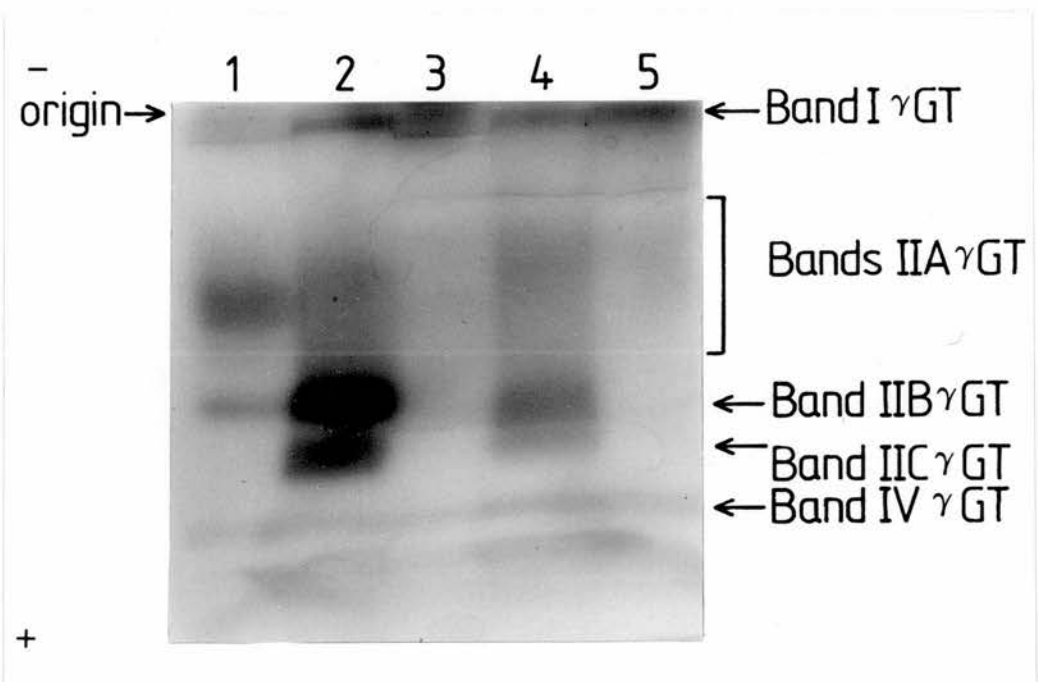


Figure 4.23 Polyacrylamide slab gel electrophoresis of serum from a patient with alcoholic cirrhosis after incubation with increasing concentrations of glycochenodeoxycholate (GC), or with saline as a control. 1, saline; 2, 10 µmol/l GC; 3, 50 µmol/l GC; 4, 100 µmol/l GC; 5, 250 µmol/l GC; 6, 500 µmol/l GC; 7, 1 mmol/l GC; 8, 2.5 mmol/l GC; 9, 5 mmol/l GC; 10, 10 mmol/l GC.

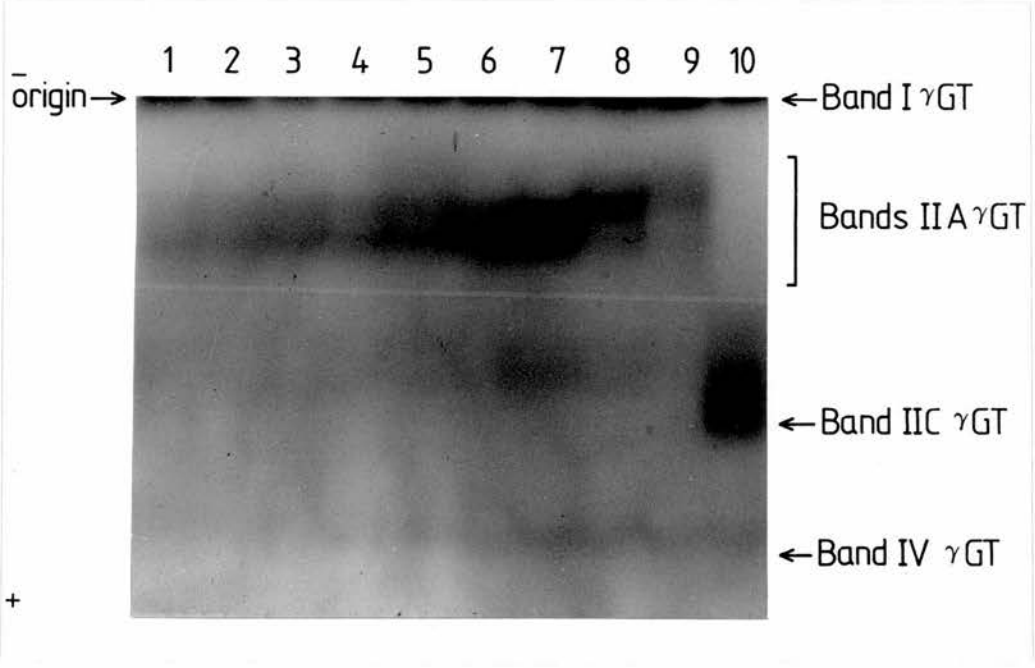
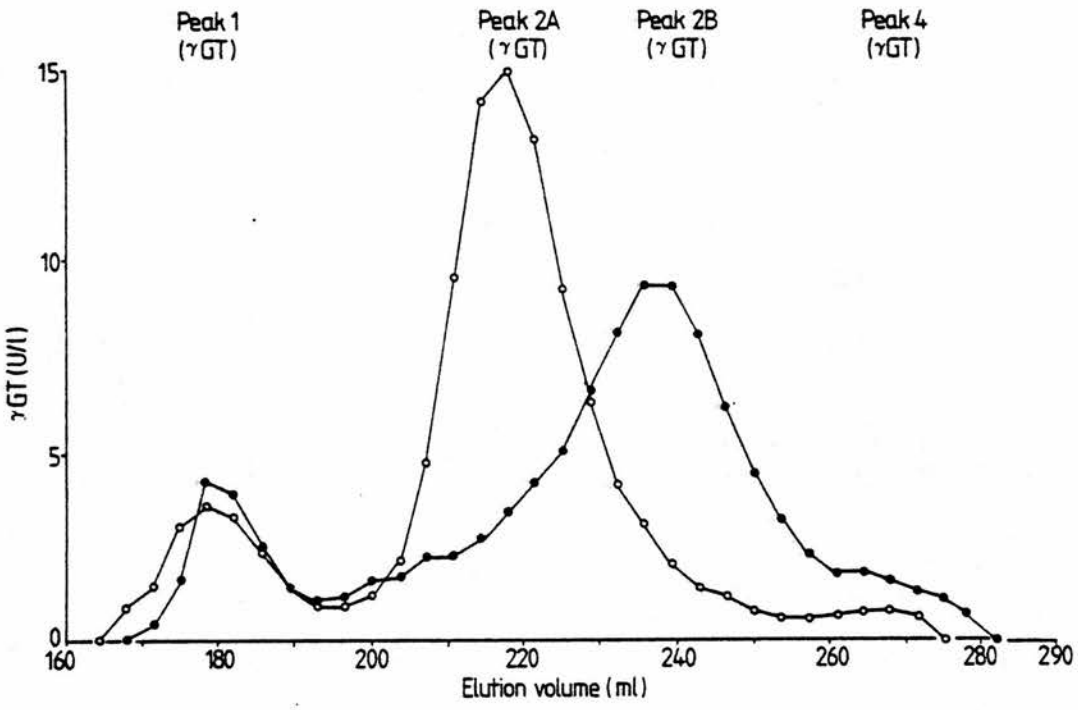


Figure 4.24 Gel chromatography on Sephacryl S300 of serum from an alcoholic patient before and after incubation with an equal volume of 20 mmol/l glycochenodeoxycholate. ○ , before; ● , after.



4.10 EFFECT OF DIALYSIS UPON THE ELUTION PROFILE OF γGT IN HUMAN SERUM

This investigation was carried out to see if there was some dialysable factor, present in sera from patients with extrahepatic biliary obstruction, which was responsible for Peak 2A (γGT) being present as Peak 2B (γGT).

A sample of serum (1 ml) was dialysed overnight at 4°C against 1000 ml of Tris-HCl buffer, pH 8.0, and for a further 12 h at 4°C after a change of buffer. The original and the dialysed serum were then subjected to gel chromatography on Sephacryl S300 in the absence of bile salts. No change in the elution profile of γGT was observed as a result of prior dialysis of the serum (Fig. 4.25).

4.11 EFFECT OF FREEZING AND THAWING

The stability of the various γGT peaks to freezing and thawing was studied by freezing a serum sample to -60°C, thawing at 37°C, and repeating the process three times. Gel chromatography was performed on the original serum before and after treatment by the freezing and thawing procedure. The elution profile of the various fractions was unaffected by the freezing and thawing procedure (Fig. 4.26).

4.12 STUDIES UPON THE FRACTIONS OBTAINED AFTER GEL CHROMATOGRAPHY

4.12.1 Peak 4 (γGT)

Samples of serum containing 0.24 U of γGT activity were added to 0.5 ml of concentrated Peak 4 (γGT) eluate, also containing 0.24 U of γGT activity. The volumes were made up to 2 ml with Tris-HCl buffer, pH 8.0, and incubated for 6 h at 37°C. Gel chromatography on a 65 x 26 cm column of Sephadex G200 revealed that approximately 86% of the added Peak 4 (γGT)

Figure 4.25 Elution profile on Sephacryl S300 of γGT in the serum of a patient with extrahepatic biliary obstruction before and after dialysis against 20 mmol/l Tris-HCl buffer pH 8.0. ○ , before dialysis; ● , after dialysis.

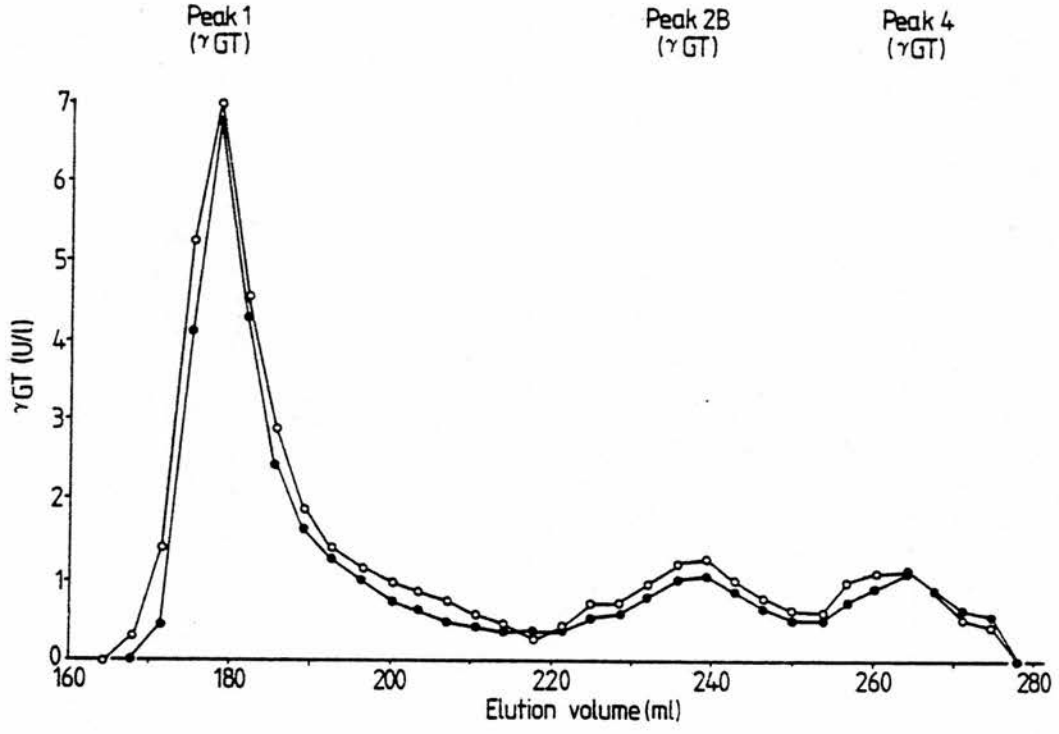
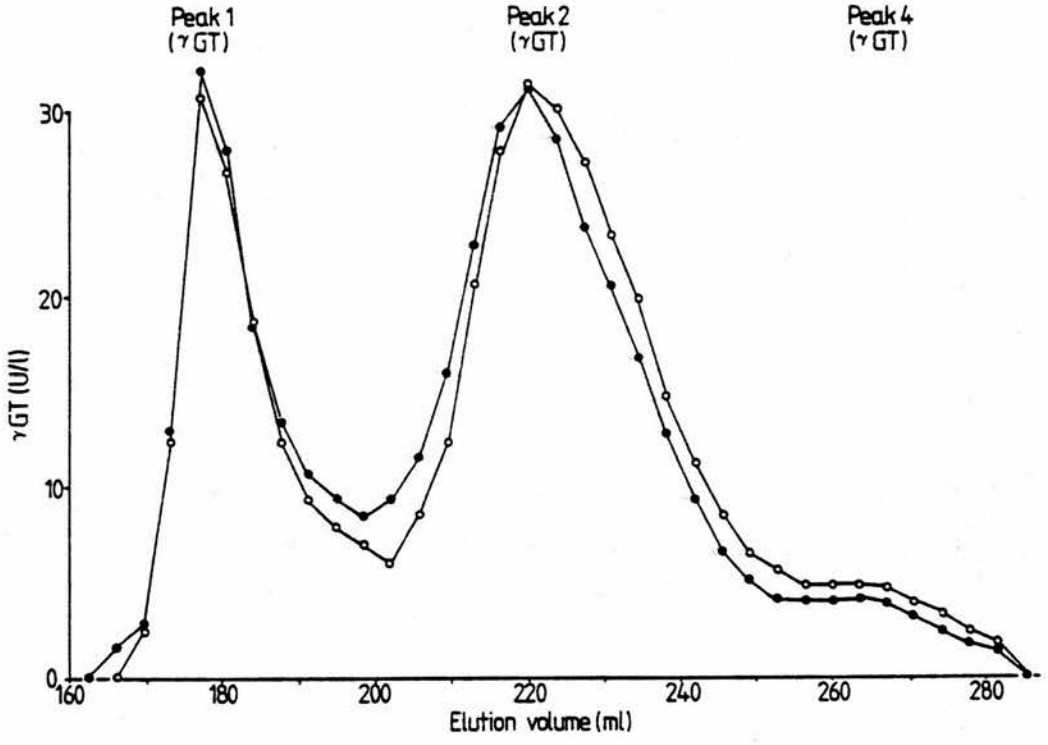


Figure 4.26 Elution profile on Sephacryl S300 of γGT in serum before ○ , and after ● , repeated freezing and thawing.



activity was recovered as Peak 4 (γGT) (Fig. 4.27). This suggests that the incubation procedure did not alter the chromatographic behaviour of Peak 4 (γGT).

4.12.2 Peak 3 (γGT)

Gel chromatography on Sephacryl S300 was performed on a patient's serum containing Peak 2A (γGT) and 2B (γGT), in the presence of 12 mmol/l deoxycholate. The Peak 3 (γGT) which was obtained was pooled, concentrated and dialysed against 20 mmol/l Tris-HCl buffer, pH 8.0.

Gel chromatography on Sephacryl S300 of 1 ml of this concentrated pool in the presence or absence of bile salts, revealed that, in the absence of bile salts, Peak 3 (γGT) reaggregates (Fig. 4.28).

The pool representing the combined Peaks 2A (γGT) and 2B (γGT) was incubated overnight at room temperature with an equal volume of normal serum. This mixture, the pool of Peaks 2A (γGT) and 2B (γGT), and the original serum from which these peaks were derived were then incubated overnight with antiserum to apolipoprotein A and with saline as a control. The mixtures were subjected to electrophoresis on 4 - 30% polyacrylamide gradients and stained for γGT activity. When compared to a saline control, a decrease was observed in enzyme staining in the areas corresponding to Bands IIA (γGT) and Band IIB (γGT) in all of the incubations (Fig. 4.29).

Similarly, the concentrated Peak 3 (γGT) pool was incubated with an equal volume of normal serum overnight at room temperature. This mixture and the Peak 3 (γGT) pool were incubated with antiserum to apolipoprotein A or with a saline control, and subjected to electrophoresis. On staining for γGT activity, a decrease in staining intensity of the zones of intermediate mobility was observed only in the Peak 3 (γGT) - normal serum mixture, as compared to a saline control (Fig. 4.30).

Figure 4.27 Elution profile on Sephadex G200 of γGT in serum from a patient with extrahepatic biliary obstruction before ○ , and after ● , incubation with Peak 4 (γGT).

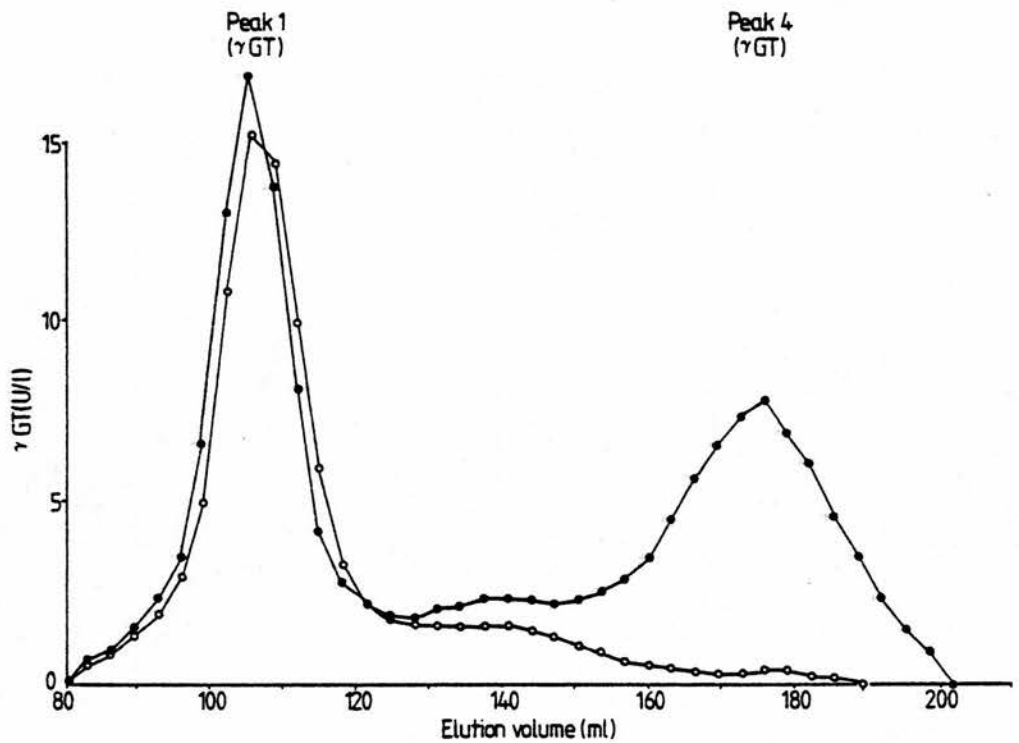


Figure 4.28 Rechromatography of Peak 3 (γGT) on Sephacryl S300 with ○ , or without ● , 12 mmol/l deoxycholate in the elution buffer.

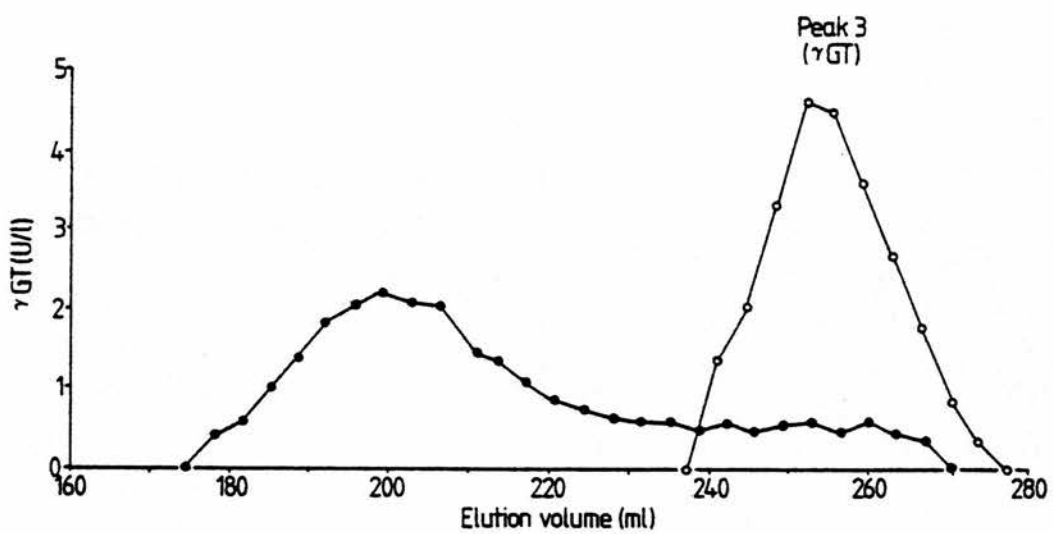


Figure 4.29 Polyacrylamide gradient gel electrophoresis of a patient's serum, a pool of Peaks 2A and 2B (γGT) and mixture composed of equal proportions of the pool and normal serum, before and after incubation with antiserum to apolipoprotein A. Serum, 1, before, 2, after; pool, 3, before, 4 after; pool/normal serum mixture, 5, before, 6, after.

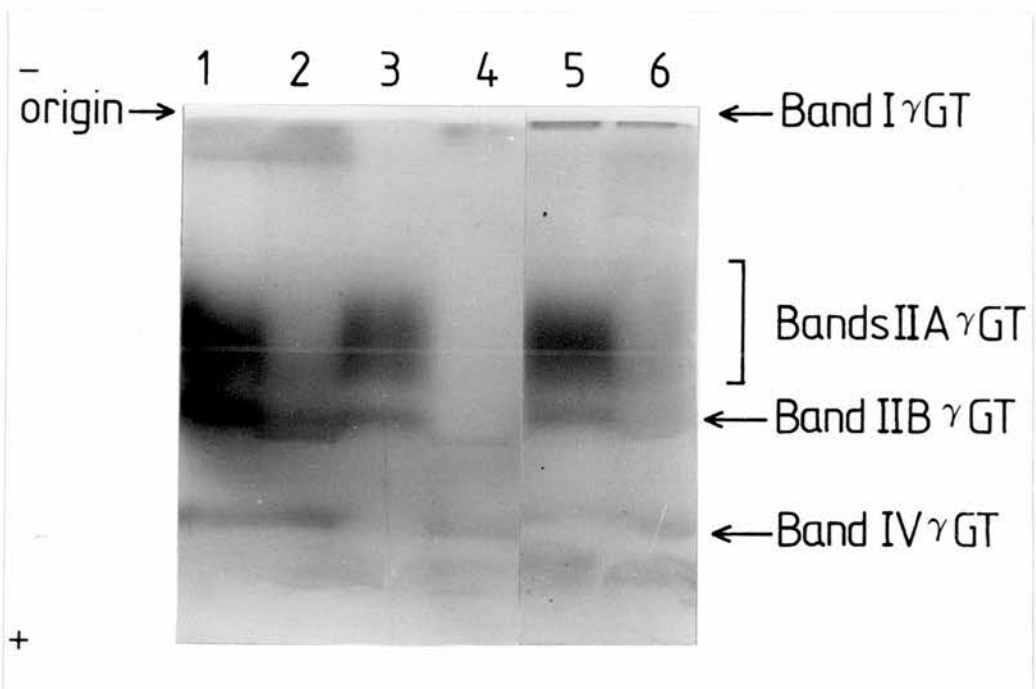
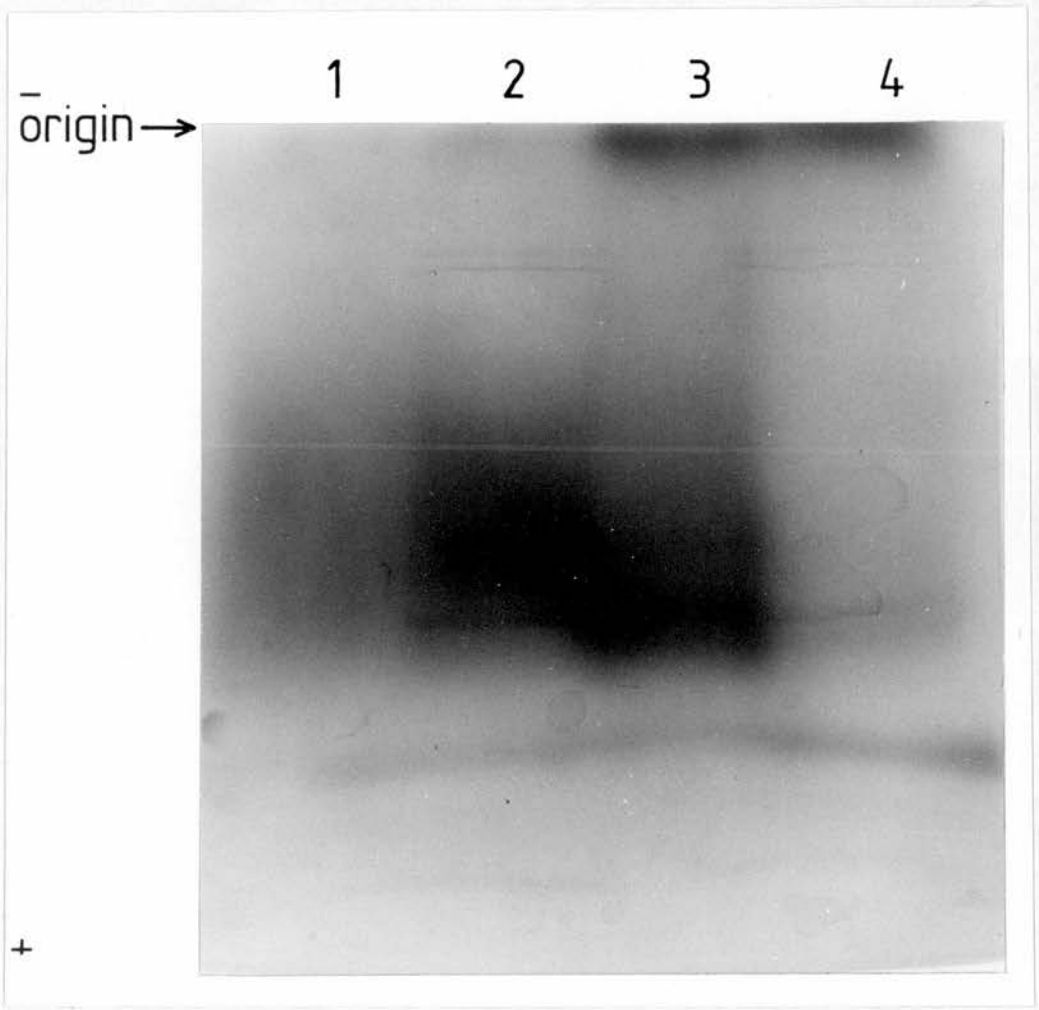


Figure 4.30 Polyacrylamide gradient gel electrophoresis of a Peak 3 (γGT) pool and a Peak 3 (γGT)/normal serum mixture, before and after incubation with antiserum to apolipoprotein A. Peak 3 (γGT) pool, 1, before; 2, after; Peak 3 (γGT) pool /normal serum mixture, 3, before; 4, after.



4.13 CATALYTIC PROPERTIES

Since there is a distinct possibility that the γGT fractions obtained from serum and bile (Chapter 3) might be related, a brief comparison of their kinetic properties was undertaken.

The reaction catalysed by γGT follows a ping-pong bi-bi kinetic mechanism (see section 1.1.3). The most accurate way of estimating the K_m values for each substrate is by replotting rate data obtained at several concentrations of the second substrate. Since the objective of this brief study however, was to compare directly the kinetic properties of the various γGT fractions in human bile and serum, the simple experimental design of Echetebu and Moss (1982a) using only a single, high concentration of second substrate was used.

4.13.1 Michaelis constants

In the analysis of enzyme kinetics, the estimates of K_m and V_{max} were obtained by a least-squares non-linear fitting procedure of the direct velocity versus substrate concentration data points (Wilkinson, 1961). The calculations were programmed on a Hewlett-Packard 9821 Desk Calculator.

γ-L-Glutamyl-p-nitroanilide

The K_m values for the donor substrate were determined at a constant concentration of 120 mmol/l glycylglycine in the final reaction mixture, while the concentration of donor substrate was varied from 1 - 9 mmol/l.

Glycylglycine

Acceptor substrate K_m values were similarly determined at a constant concentration of donor substrate of 9 mmol/l, while varying the concentration of acceptor substrate from 10 to 120 mmol/l. The K_m values obtained in the serum and bile fractions are shown in Table 4.6. The differences observed in the K_m values were not statistically significant.

Table 4.6

Michaelis constants of the various γGT fractions obtained from serum and bile

Fraction (n)	Michaelis Constant (± S.D.)	
	Glycylglycine	γ-L-Glutamyl-p-nitroanilide
Serum (7)	16.39 ± 2.20	1.54 ± 0.22
Bile (4)	15.91 ± 2.20	1.42 ± 0.14
Serum Peak 1 (4)	16.00 ± 3.23	1.77 ± 0.38
Serum Peak 2A (5)	16.40 ± 3.23	1.67 ± 0.37
Serum Peak 2B (4)	16.74 ± 3.80	1.42 ± 0.24
Serum Peak 4 (3)	13.42 ± 1.66	1.34 ± 0.16
Bile Peak 1 (2)	15.81 ± 3.40	1.62 ± 0.37
Bile Peak 4 (5)	15.40 ± 1.70	1.36 ± 0.40

4.13.2 Inhibitor studies

Glycine was used for inhibitor studies. This amino acid is believed to interact with the enzyme by behaving as a substrate analogue for both donor and acceptor substrates, resulting in dead-end inhibition (Stromme and Theodorsen, 1976). With donor substrate the inhibition is competitive, whilst with acceptor substrate it is uncompetitive (Stromme and Theodorsen, 1976). Replots of the slopes and the intercepts of the Lineweaver-Burke plots obtained from the initial kinetic data were used to determine the inhibition constants. Each Lineweaver-Burke plot was obtained at different inhibitor concentrations in the final reaction mixture ranging from 0 to 20 mmol/l. For the competitive inhibition observed on the donor substrate the replot was K_m/V_{max} versus inhibitor concentration. For the uncompetetive inhibition observed on the acceptor substrate the replot was $1/V_{max}$ versus inhibitor concentration.

The fitted lines in the replots were obtained by least-squares linear regression performed on a Hewlett-Packard 9821 Desk Calculator, which also calculated the intercept (and standard error) of the line on the inhibitor concentration axis, thus giving the value of the inhibitor constant. The values obtained for the inhibitor constants of the various fractions are shown in Table 4.7. The differences between the values of each inhibitor constant for the various fractions were not statistically significant.

Table 4.7

Inhibitor constants for glycine of the γGT fractions obtained from serum and bile

Fraction	Inhibitor Constant ± (S.E.)	
	Glycylglycine	γ-L-Glutamyl-p-nitroanilide
Serum	25.12 ± 3.21	4.03 ± 0.092
Bile	22.23 ± 2.44	4.73 ± 0.60
Serum Peak 1	22.60 ± 4.73	4.51 ± 0.68
Serum Peak 2A	22.47 ± 2.53	4.42 ± 0.40
Serum Peak 2B	27.36 ± 4.12	4.33 ± 0.35
Serum Peak 4	21.72 ± 3.02	3.75 ± 0.16
Bile Peak 1	21.64 ± 3.99	4.43 ± 0.95
Bile Peak 4	28.80 ± 2.11	3.92 ± 0.61

The results suggest that there is no difference in the catalytic properties studied, between the different γGT fractions obtained from serum and bile. The significance of this finding is considered in Chapter 7 of this thesis.

4.14 STUDIES ON LEUCINE AMINOPEPTIDASE AND ALKALINE PHOSPHATASE

4.14.1 Effect of papain treatment

Variable amounts of LAP and ALP activity were destroyed following papain treatment (Table 4.8).

Table 4.8

Enzyme activities after digestion of sera with papain

Sample	Percentage initial enzyme activity	
	LAP	ALP
Serum A	81	74
Serum B	91	64
Serum C	66	57

4.14.2 Gel chromatography performed in the absence of bile salts

After gel chromatography of normal sera and sera from patients with liver disease, varying amounts of LAP eluted as Peaks 1, 2 and 4 (LAP) (Table 4.9). In contrast to Peak 2 (γGT), no splitting of Peak 2 (LAP) was noticed although it appeared to constitute a smaller proportion of total activity in patients with extrahepatic biliary obstruction as compared to the rest (Figs. 4.31 and 4.32). In contrast to both γGT and LAP, ALP showed no intermediate M_r peaks (Figs. 4.31 and 4.32) (Table 4.9). The recovery of enzyme activity was variable; for LAP mean 104%, range 81 - 155%; for ALP mean 105.3%, range 82 - 143%.

Figure 4.31 Elution profiles of LAP and ALP following gel chromatography on Sephacryl S300 of serum from a patient with extrahepatic biliary obstruction. ● , LAP; ○ , ALP.

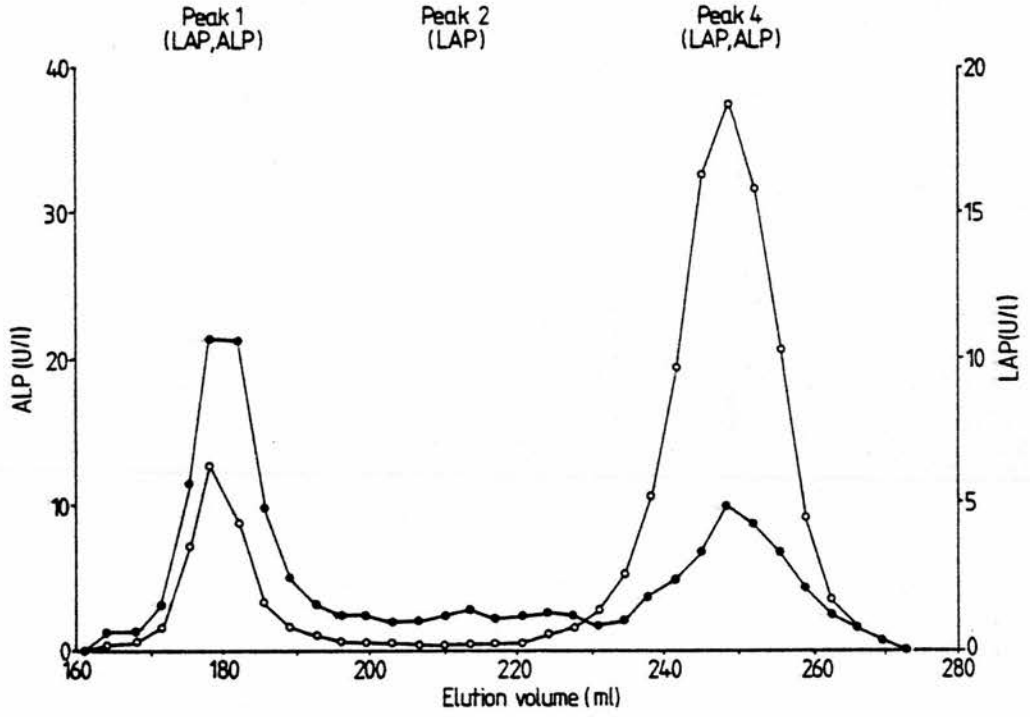


Figure 4.32 Gel chromatography on Sephacryl S300 of LAP and ALP in serum from a patient with alcoholic cirrhosis. ● , LAP; ○ , ALP.

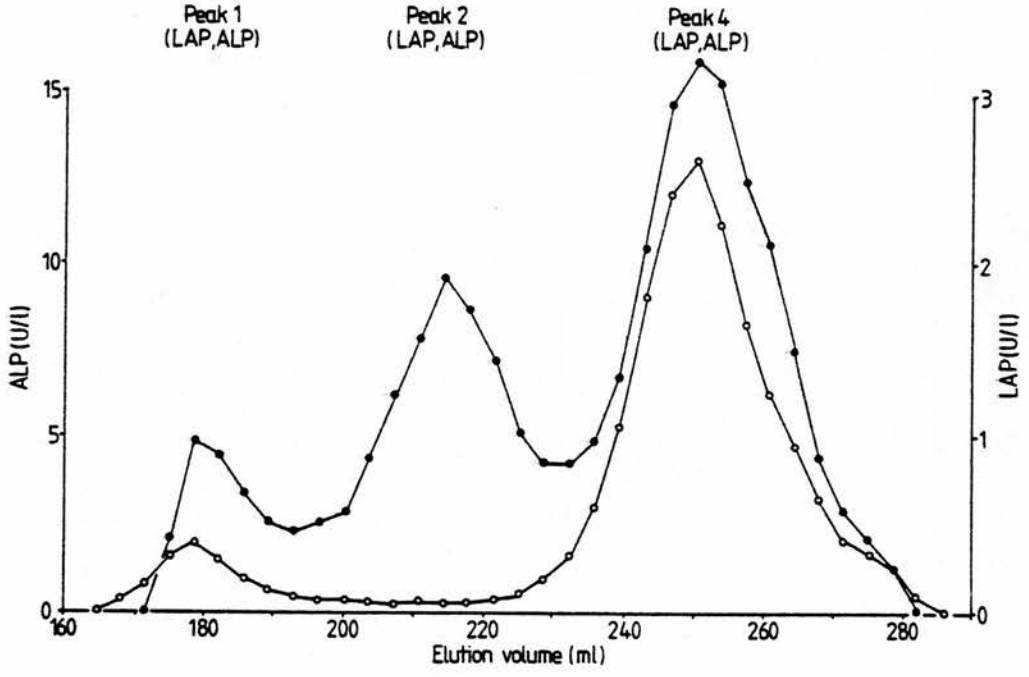


Table 4.9

Distribution of LAP and ALP activity obtained after gel chromatography of 110 sera on Sephacryl S300

Fraction	Percentage of total recovered activity	
	Mean	Range
Peak 1 (LAP)	18.5	3 - 60
Peak 2 (LAP)	22.5	0 - 53
Peak 4 (LAP)	59	27 - 91
Peak 1 (ALP)	14.0	2 - 54
Peak 4 (ALP)	86	46 - 98

4.14.3 Gel chromatography performed in the presence of bile salts

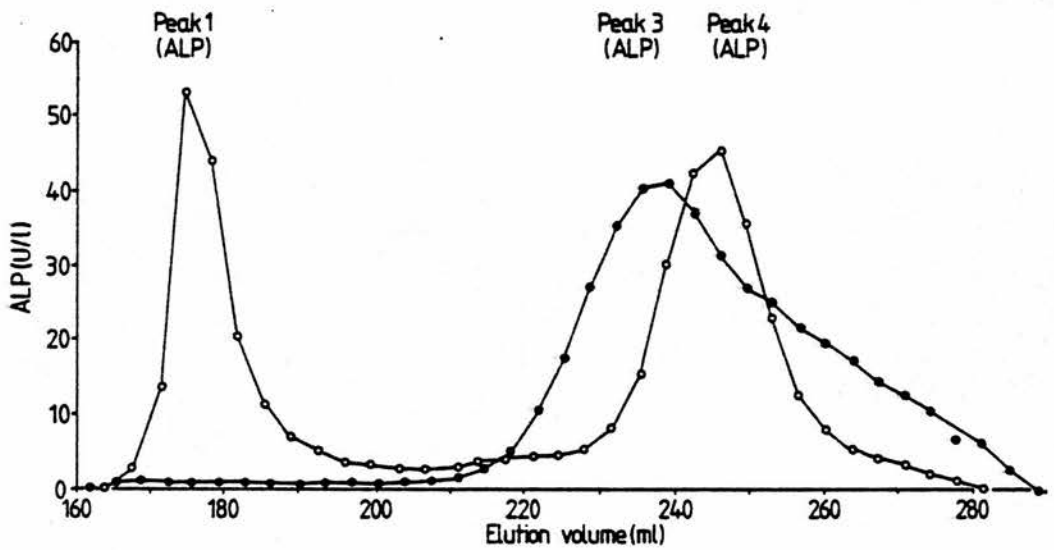
When serum was chromatographed in the presence of 12 mmol/l deoxycholate or glyochenodeoxycholate, the amount of ALP activity eluting as Peak 1 (ALP) decreased, together with the appearance of activity eluting as Peak 3 (ALP) (Fig. 4.33). Although the elution volume of Peak 4 (ALP) appeared to be unaffected, there was a suggestion of some loss of activity.

When a concentrated pool of Peak 1 (ALP) was rechromatographed in the presence of bile salts it disappeared, giving rise to Peak 3 (ALP). No LAP activity was ever recovered in the eluate when serum or Peak 1 (LAP) were chromatographed in the presence of bile salts.

4.14.4 Gel chromatography of papain-treated serum

Gel chromatography of papain-treated serum in the absence of bile salts resulted in a decrease in LAP activity eluting as Peaks 1 and 2 (LAP) together

Figure 4.33 Elution profile on Sephacryl S300 of ALP with ● , or without ○ , 12 mmol/l deoxycholate in the elution buffer.



with an increase in activity eluting as Peak 4 (LAP) (Fig. 4.34). There was also a decrease in activity of Peak 1 (ALP) but no concomitant increase in activity associated with Peak 4 (ALP).

The estimated $\underline{M}_r$ values of the enzyme fractions obtained after gel chromatography are shown in Table 4.10.

Table 4.10

Estimated $\underline{M}_r$ values of the enzyme fractions obtained following gel chromatography

Fraction	Estimated $\underline{M}_r$	
	LAP	ALP
Peak 2	400 000 - 6 10 000	**
Peak 3	*	280 000
Peak 4	190 000	200 000

* Not estimable

** Not present

4.14.5 7% polyacrylamide gel electrophoresis

Results obtained for LAP were analogous to those described for γGT. Three main areas of activity were observed; one at the origin (Band 1 (LAP)), one of varying mobility (between 5 and 35% of that of albumin) (Bands II (LAP)) and two bands with mobility approximately 55 and 60% of that of albumin (Band IVA (LAP) and Band IVB (LAP)) (Fig. 4.35). Bands IVA and IVB (LAP) were also obtained following electrophoresis of the fraction corresponding to Peak 4 (LAP) obtained after gel chromatography.

Figure 4.34 Gel chromatography of LAP in human serum before ○ , or after ● , treatment with papain.

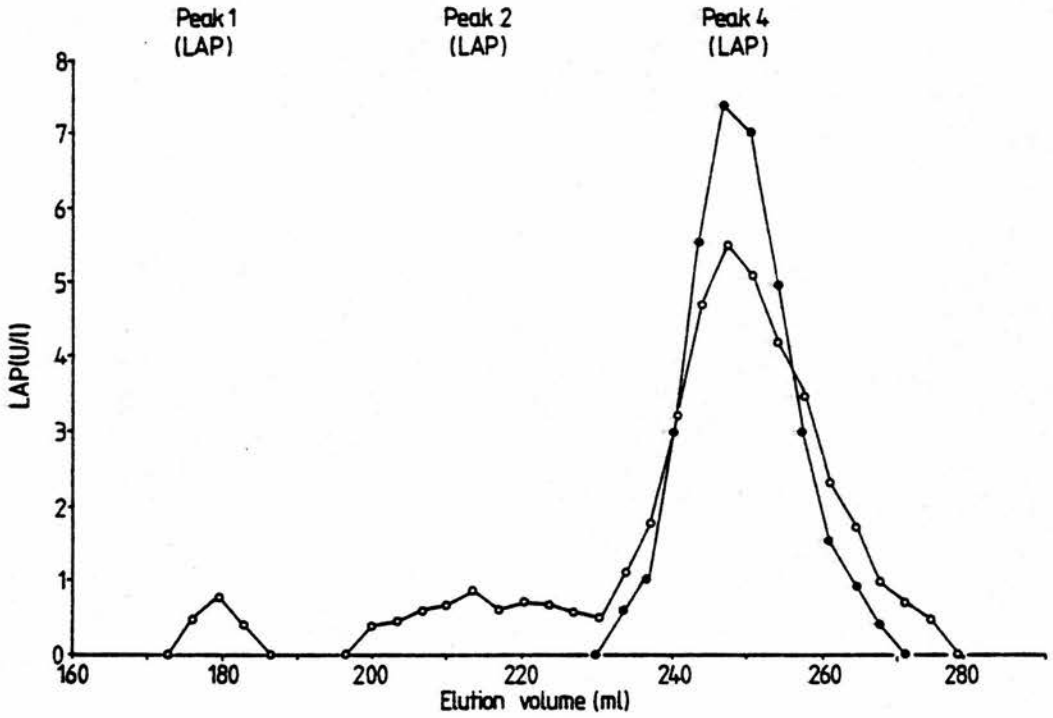
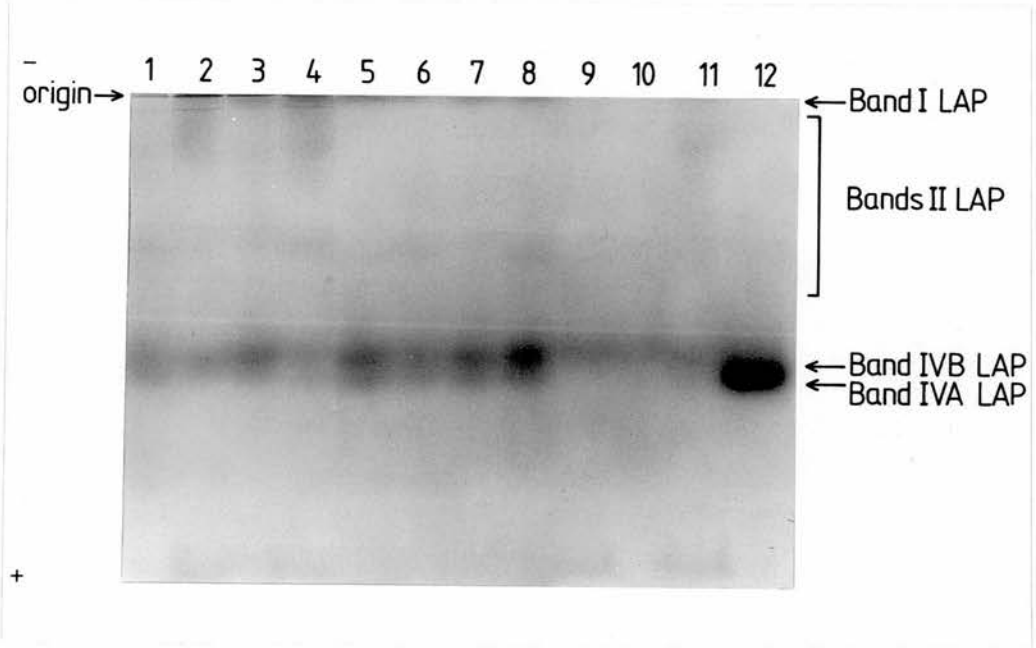


Figure 4.35 Polyacrylamide slab gel electrophoresis of sera from patients with liver disease and a papain-treated serum, stained for LAP activity. 1 - 4, primary biliary cirrhosis; 5 - 8, extrahepatic biliary obstruction; 9 - 11, chronic active hepatitis; 12, papain-treated serum.



Electrophoresis of papain-treated sera yielded a single band of LAP activity of equal mobility to Band IVB (LAP) (Fig. 4.35).

4.14.6 Polyacrylamide gradient gel electrophoresis

The estimated $\underline{M}_r$ values of the various fractions obtained are shown in Table 4.11.

Table 4.11

Estimated $\underline{M}_r$ values of the various enzyme fractions obtained after gel chromatography as determined by gradient gel electrophoresis

	Estimated $\underline{M}_r$	
Fraction	LAP	ALP
Peak 2	*	***
Peak 3	**	260 000
Peak 4	170 000	180 000

* The staining of this fraction was too faint and diffuse to allow an accurate estimation of $\underline{M}_r$.

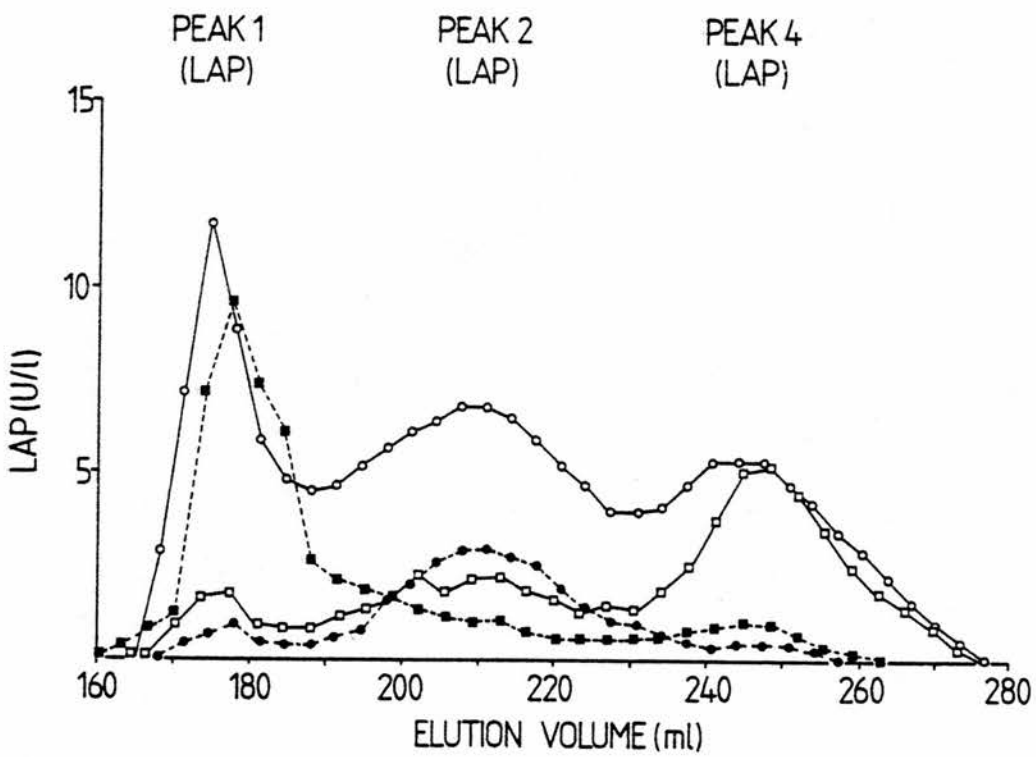
** Not estimable

*** Not present

4.14.7 Polyanion precipitation

Results were obtained for LAP, analogous to those obtained for γGT (see section 4.6). Peak 1 (LAP) co-precipitated with LDL and VLDL and Peak 2 (LAP) co-precipitated with HDL (Fig. 4.36). ALP activity as Peak 1 (ALP), however, only precipitated with the VLDL and LDL fraction.

Figure 4.36 Elution profile of LAP in serum and the lipoprotein fractions from a patient with gallstones before and after precipitation with polyanions. ○ , whole serum; ■ , LDL and VLDL; ● , HDL; □ , supernatant after precipitation of LDL, VLDL and HDL.



4.14.8 Incubation with antisera

Sera were incubated with antiserum to apolipoprotein A and with saline as a control (see section 4.7.3) and subjected to electrophoresis. On staining for LAP activity there was a decrease in the staining intensity of the area of intermedate mobility (Fig. 4.37).

4.14.9 Effect of dialysis and freezing and thawing

Dialysis against 20 mmol/l Tris-HCl, pH 8.0 buffer, or repeated freezing to -60°C followed by thawing at 37°C (4 times), had no effect upon the elution patterns of the enzymes from the Sephacryl column (Fig. 4.38).

4.15 DISCUSSION

The results presented in this chapter confirm previous reports that γGT is present in normal sera, and in the sera of patients with liver disease as high, intermediate and low M_r forms (Orlowski et al., 1965; Kokot and Kuska, 1968; Huseby, 1978; Wenham et al., 1979; Echetebu and Moss, 1982a). They have also shown that another hepatocyte plasma membrane enzyme, LAP, is present in high, intermediate and low M_r forms, unlike ALP which does not possess the intermediate M_r form.

The high M_r form of γGT (and also that of LAP and of ALP) co-precipitated with VLDL and LDL after treatment with polyanions. This is consistent with previous reports that these high M_r forms may consist of complexes of the enzymes with lipoproteins or lipoprotein-X (Price and Sammons, 1974; Wenham et al., 1979; Crofton and Smith, 1981b; Huseby, 1982a).

On the other hand, varying amounts of the intermediate M_r forms of γGT (and also of LAP), with the notable exception of Peak 2B (γGT) co-precipitated with HDL; an observation in agreement with that of Huseby (1982a). In contrast, all of the intermediate M_r γGT (and LAP) including Peak 2B (γGT), were shown to bind to antiserum to apolipoprotein A but not to apolipoprotein

Figure 4.37 Polyacrylamide slab gel electrophoresis of sera from patients with liver disease after incubation 1 and 3, with saline; or 2 and 4, with antiserum to apolipoprotein A. 5 antiserum to apolipoprotein A.

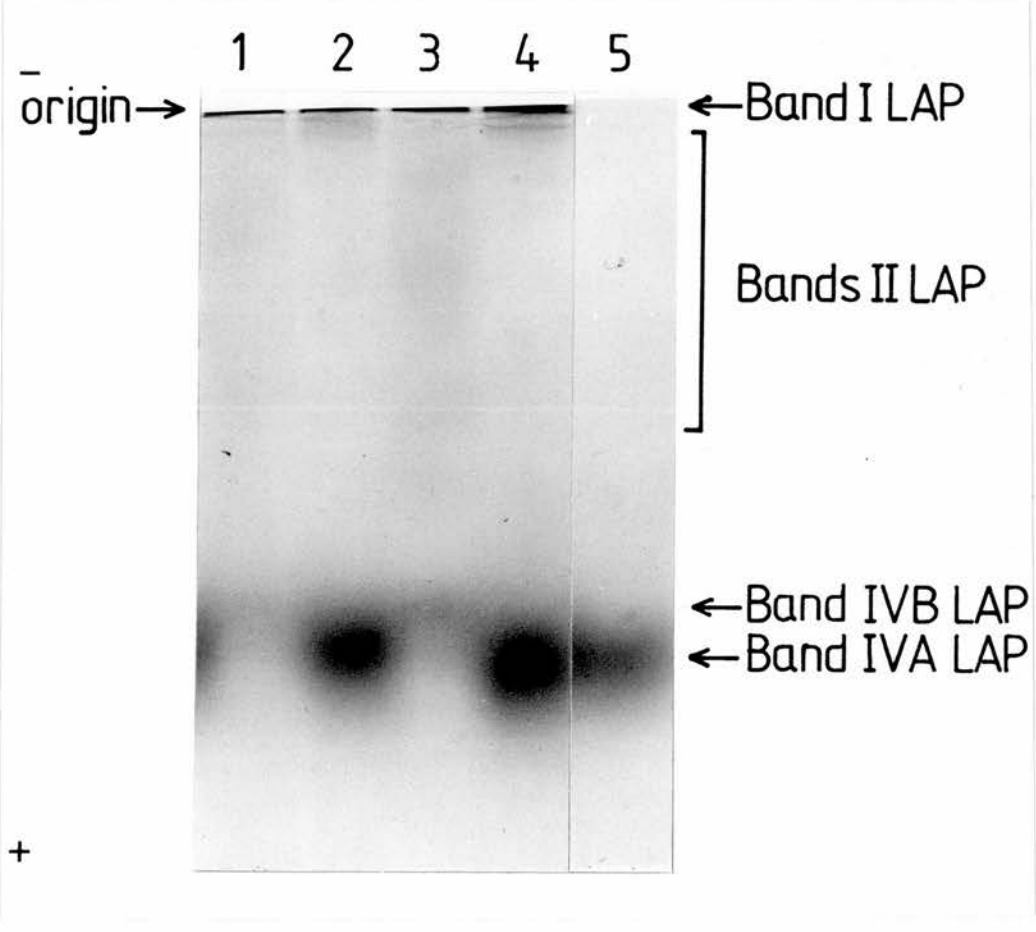
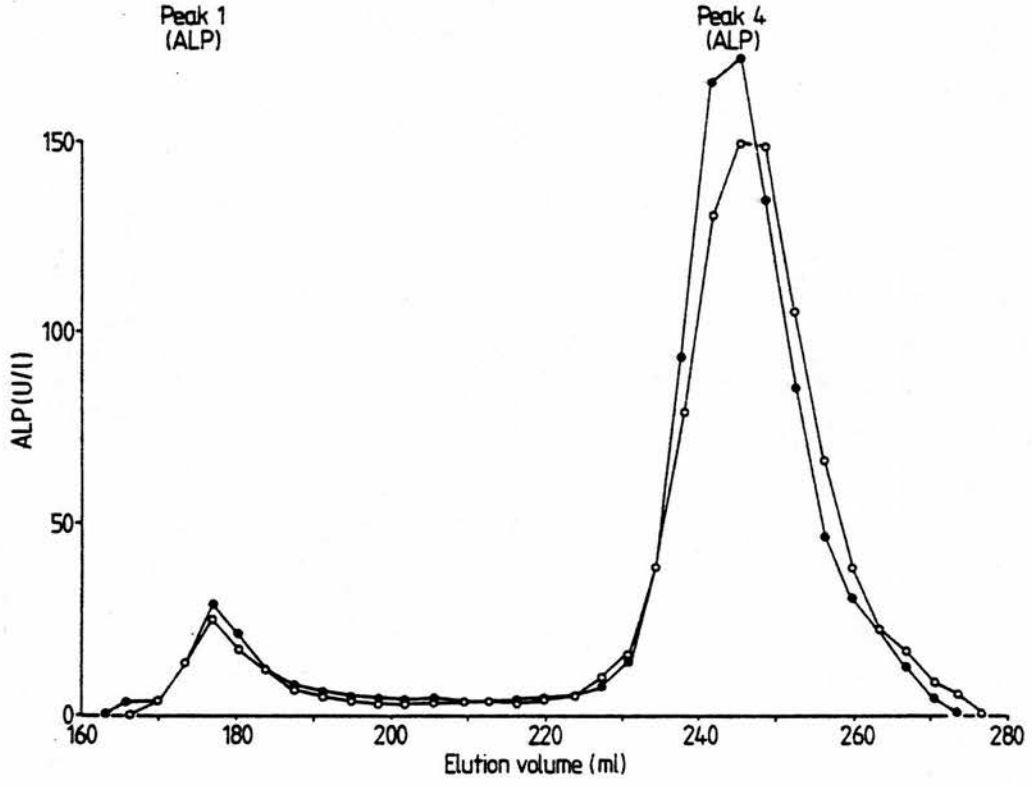


Figure 4.38 Elution profile on Sephacryl S300 of ALP in serum before ○ , and after ● , repeated freezing and thawing.



B. The amount of γGT activity precipitated by the antiserum to apolipoprotein A was variable, a finding in agreement with that of previous workers (Huseby, 1982a; Artur et al., 1984a, 1984b). The latter authors, however, were also able to precipitate γGT activity using antiserum to apolipoprotein B, a finding at variance with the results both of the present study and those of Huseby (1982a). This apparent discrepancy could be due to the presence of antibody directed against γGT protein, in their antiserum to low density lipoprotein, a property that has already been ascribed to antiserum to lipoprotein-X (Huseby, 1982a). The results described in this chapter strongly suggest that the intermediate $\underline{M}_r$ forms of γGT (and of LAP) consist of complexes of the enzyme with HDL. The $\underline{M}_r$ of an HDL particle may range from 175 000 to 350 000 (Lewis, 1976; Huseby, 1982a), and so the estimated $\underline{M}_r$ of the intermediate $\underline{M}_r$ γGT (and LAP) is consistent with the attachment of a single molecule of γGT (or of LAP) onto such an HDL particle. However, the possible attachment of more than one enzyme molecule per HDL particle is not precluded in view of the wide range of $\underline{M}_r$ observed, particularly in Peak 2A (γGT). As well as co-precipitating with HDL, up to 30% of the intermediate $\underline{M}_r$ γGT also co-precipitated with LDL and VLDL. There are two possible explanations for this; firstly, that the very presence of γGT on the HDL complex alters the behaviour of this complex when treated with a given concentration of polyanions, and secondly, that γGT is associated with LDL or VLDL or both. The latter possibility is rendered unlikely by the fact that no γGT activity was precipitated by antiserum to apolipoprotein B. The reason why Peak 2B (γGT) did not precipitate with polyanions is unclear. Although there is no evidence, it is possible that the precipitation characteristics of these particles may have been altered by the presence of high concentrations of bile salts in the serum of these patients.

The mean bile salt concentrations of serum from patients with extrahepatic biliary obstruction were over five times those of the other groups. An attempt was therefore made to mimic the abnormalities found in serum in obstructive jaundice, by incubating sera that contained Peak 2A (γGT), with hepatic bile. This incubation resulted in the conversion of Peak 2A (γGT) to a peak with an elution pattern similar to that of Peak 2B (γGT). The ability of bile to effect this change was a) inhibited by prior dialysis of the bile and b) reproduced by incubation with glycochenodeoxycholate. These results suggest that the

conversion of Peak 2A (γGT) to Peak 2B (γGT) was caused by the bile salts present in the bile. However, electrophoresis of the bile / bile salt / sera mixtures showed the presence of Band IIC (γGT) which was not previously detected in any of the sera. This suggests that bile salt concentrations are not the only factor involved in the formation of Peak 2B (γGT) in the sera of patients with obstructive jaundice. This hypothesis is supported by the fact that dialysis appears to have no effect upon Peak 2B (γGT), either in terms of its $\underline{M}_r$ or total recovered activity.

The elution pattern of γGT (and ALP) changed markedly when gel chromatography was performed in the presence of bile salts, as most of the activity eluted as the Peak 3 forms. The picture is incomplete, however, since bile salts inhibited LAP activity. The Peak 3 forms were hydrophobic in nature and readily reaggregated if the bile salts were removed by dilution or dialysis. The Peak 4 form of γGT (and also of LAP and of ALP) on the other hand, observed in untreated sera, or obtained following treatment with papain, was hydrophilic in nature and showed no tendency to reaggregate after incubation with serum.

The inability of antiserum to apolipoprotein A to bind Peak 3 (γGT) suggests that the γGT-HDL complex had either been dispersed or radically altered in its properties. In an attempt to reform the complex, Peak 3 (γGT) was incubated with normal serum. When this had been done the antiserum appeared to be able to bind to the γGT in the serum - Peak 3 (γGT) mixture. This phenomenon suggests that the γGT-HDL interaction is hydrophobic in nature and that it can form spontaneously in serum <u>in vitro</u>. The complex also appears to have a stable physical configuration since freezing and thawing had no noticeable effect.

Finally, the fact that Peak 2B (γGT) is found in large amounts only in the serum from patients suffering form extrahepatic obstructive jaundice suggests that measurement of this fraction might be of clinical and diagnostic value. This hypothesis is tested and explored further in Chapter 5 of this thesis.

Chapter 5

A CLINICAL STUDY OF THE MULTIPLE FORMS OF γ-GLUTAMYLTRANSFERASE

Although it is known that γGT activity in serum is increased in patients with liver disease (Szczeklik et al., 1961; Goldbarg et al., 1963; Rosalki et al., 1971; Rosalki and Rau, 1972), most investigators have found that such measurements of total activity discriminate poorly, if at all, between the different types of liver disease. Differential measurements of the multiple forms of γGT in serum have been made much less frequently in such patients. Furthermore, in those instances where differential measurements have been made, they have usually been based on electrophoretic means of separation rather than on methods which separate the multiple forms on the basis of $\underline{M}_r$ (Jacyszyn and Laursen, 1968; Hetland et al., 1975; Kok et al., 1978; Burlina, 1978).

During the study of the physical properties of serum γGT (Chapter 4), three major categories of γGT were distinguished on the basis of $\underline{M}_r$ and electrophoretic behaviour:

1. The first fraction, high $\underline{M}_r$ γGT, which I have called Peak 1 (γGT), elutes in the void volume after gel chromatography on Sephacryl S300 and remains at the origin after polyacrylamide gel electrophoresis. This fraction is present in normal serum and tends to predominate in serum from patients with obstructive liver disease.

2. The second fraction, intermediate $\underline{M}_r$ γGT ($\underline{M}_r$ 250 000 -500 000), has an electrophoretic mobility between 8 and 55% of that of albumin. This fraction may be of two types, most easily distinguished from one another by electrophoresis. The first type separates into several bands (which I have called Bands IIA (γGT)) with mobilities between 8 and 40% of that of albumin. In contrast, the second type moves as a discrete band (Band IIB (γGT)) with a mobility of 45 - 55% of that of albumin. During the studies described in Chapter 4 it was noted that although Bands IIA (γGT) are

usually the main intermediate $\underline{M}_r$ fractions present in liver disease, Band IIB (γGT) predominates in patients with jaundice due to extrahepatic obstruction.

3. The third fraction, low $\underline{M}_r$ γGT, ($\underline{M}_r$ about 120 000) is a hydrophilic form of the enzyme. This is in contrast to the two hydrophobic forms of the enzyme mentioned above.

The present study attempts to define more precisely the physical differences (described in Chapter 4) between the γGT found in various liver diseases. In particular, it attempts to determine whether the use of γGT measurement can help to distinguish jaundice due to extrahepatic obstruction and possibly remediable by surgery, from other 'medical' causes of jaundice.

High $\underline{M}_r$ forms of leucine aminopeptidase and alkaline phosphatase also appear in serum in liver disease (Ideo and Ronchi. 1972; Price and Sammons, 1974; Crofton and Smith, 1981a). The high $\underline{M}_r$ forms of these enzymes have also been studied in order to compare their diagnostic efficiency with that of γGT. It is hoped that the results might also lead to more insight into the mechanism of release of γGT (and LAP and ALP) into the circulation.

5.1 SAMPLES USED IN THE STUDY

Sera from 100 patients with liver disease (97 of which had an elevated γGT activity) were selected from specimens sent to the department for γGT measurement. The selection of particular specimens was made on the basis of diagnosis and following discussion with the clinician looking after the patient. The diagnosis was based on clinical and laboratory findings and wherever possible confirmed by liver scan, liver biopsy or laparotomy. Sera were also obtained from 10 apparently healthy individuals with no clinical or biochemical evidence of liver damage. The classification of the patient groups, together with the abbreviations used throughout this chapter, is shown in Table 5.1.

Table 5.1

Classification of patient groups

Patient Group	No of patients	Abbreviations used in illustrations
Healthy individuals	10	Norm.
Extrahepatic biliary obstruction	25	Ob.j.
Metastatic liver disease	25	Liver 2^{o}.
Primary biliary cirrhosis	9	P.B.C.
Alcoholic cirrhosis	12	Alc. cir.
Long term anticonvulsant therapy	10	Anticonv.
Chronic active hepatitis	7	C.A.H.
Haemochromatosis	7	H'chrom.
Post liver transplant	6	T'plant

5.2 MEASUREMENT OF HIGH $\underline{M}_r$ ENZYMES

This was carried out following gel chromatography of 1 - 2 ml of serum on a 95 x 2.6 cm column of Sephacryl S300 (see section 2.3). The problem of overlapping peaks, when it arose, was overcome by extrapolation of the peaks.

The enzyme activities in the high $\underline{M}_r$ peaks were summed and the activity of the high $\underline{M}_r$ fraction calculated using the formula:-

$$\text{High } \underline{M}_r \text{ enzyme (\%)} = \frac{A}{A + B} \times 100$$

$$\text{High } \underline{M}_r \text{ enzyme (U/l)} = \frac{A}{A + B} \times \text{serum enzyme activity (U/l)}$$

Where A = The sum of the activities of the fractions in the high $\underline{M}_r$ enzyme peak.

B = The sum of all of the other recovered enzyme activity.

The recovery of enzyme activity was variable; for γGT, the mean was 101.7%, with a range of 81 - 146%; for LAP, mean 104%, range 81 - 155%; for ALP, mean 105.3%, range 82 - 143%. It is possible that these unexpected high recoveries may be attributed to the presence of inhibitors in serum which were removed or diluted during gel chromatography. The precision of the method was assessed by performing gel chromatography on a single serum sample on 11 different days and measuring the high $\underline{M}_r$ fraction each day. Coefficients of variation were 3.7% for high $\underline{M}_r$ γGT at a mean value of 58 U/l, 10.2% for high $\underline{M}_r$ LAP at a mean value of 16 U/l and 7.9% for high $\underline{M}_r$ ALP at a mean value of 22 U/l.

5.3 MEASUREMENT OF BAND IIB (γGT)

Intermediate $\underline{M}_r$ γGT was separated into its subfractions, Bands IIA (γGT) and Band IIB (γGT) by electrophoresis on 4 - 30% polyacrylamide gradient gels (see section 2.4). Polyacrylamide gradient gels were chosen instead of uniform 7% polyacrylamide slabs because:

1. They were of a more convenient size to fit the scanner.

2. Up to 4 gels, i.e. 20 samples, could be set up in each run.

3. It was more convenient to use pre-cast gels.

5.3.1 Optimal staining time of gel

The time course of colour development during the first stage of the two-step gel staining procedure was investigated to determine the optimal incubation time. Electrophoresis was performed on equal portions of a pool of Peak 2B (γGT) obtained after gel chromatography of serum on Sephacryl S300. After electrophoresis, slices of the gel corresponding to each aliquot were incubated in substrate solution for various lengths of time. At the end of each period the

gels were immersed in a solution of Fast Blue B diazonium salt in 10% (v/v) acetic acid for 15 min then scanned at 525 nm using an Auto Scanner Flur-Vis (Helena Laboratories, Beaumont, Texas, USA). The results (Fig. 5.1) show that the increase in colour development (as measured by the area under the peak) was rectilinear with time up to 6 h. Three hours was arbitrarily chosen as a convenient incubation time.

5.3.2 Variation of staining intensity with amount of enzyme applied to gel

Samples (0 - 40 µl) of a concentrated pool of Peak 2B (γGT) were made up to 50 µl with saline, then subjected to polyacrylamide gradient gel electrophoresis. The gels were then incubated with substrate for 3 h, stained for 15 min in Fast Blue B-glacial acetic acid and then scanned. The graph of colour intensity versus amount of enzyme (volume of Peak 2B (γGT)) electrophoresed was linear up to an applied activity of 8.4×10^{-3} U (Fig. 5.2). The γGT activity of the pool was 2 10 U/l.

5.3.3 Calculation and precision of the method

Band IIB (γGT) was estimated as a percentage of the total intermediate $\underline{M}_r$ γGT from the formula:

$$\text{Band IIB } (\gamma\text{GT}) = \frac{C}{C + D} \times 100$$

Where C = The area of the peak corresponding to Band IIB (γGT) obtained after scanning.

Where D = The area of the peaks corresponding to Bands IIA (γGT) obtained after scanning.

Between-batch precision was estimated by performing electrophoresis on 10 sera on 2 different occasions, each on different days. A coefficient of variation of 11.2% was obtained for Band IIB (γGT) at a mean of 55% (range 0 - 100%). The mean serum γGT activity was 309 U/l (range 28 - 625 U/l).

Figure 5.1 Electrophoresis of Peak 2B (γGT) on polyacrylamide gradient gel. Variation in intensity of Band IIB (γGT) with incubation time.

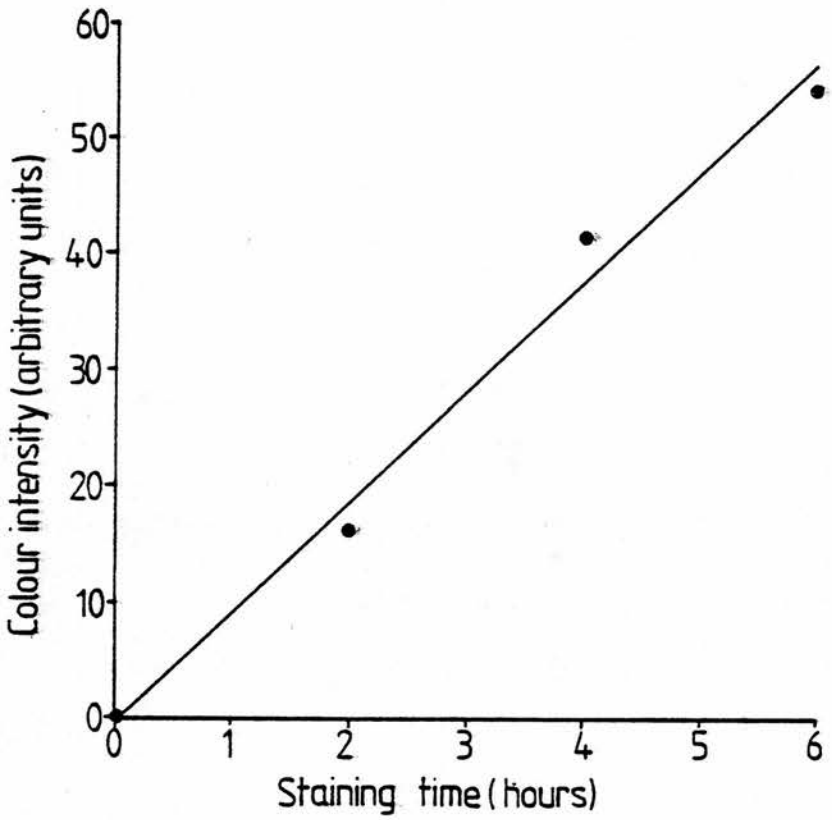
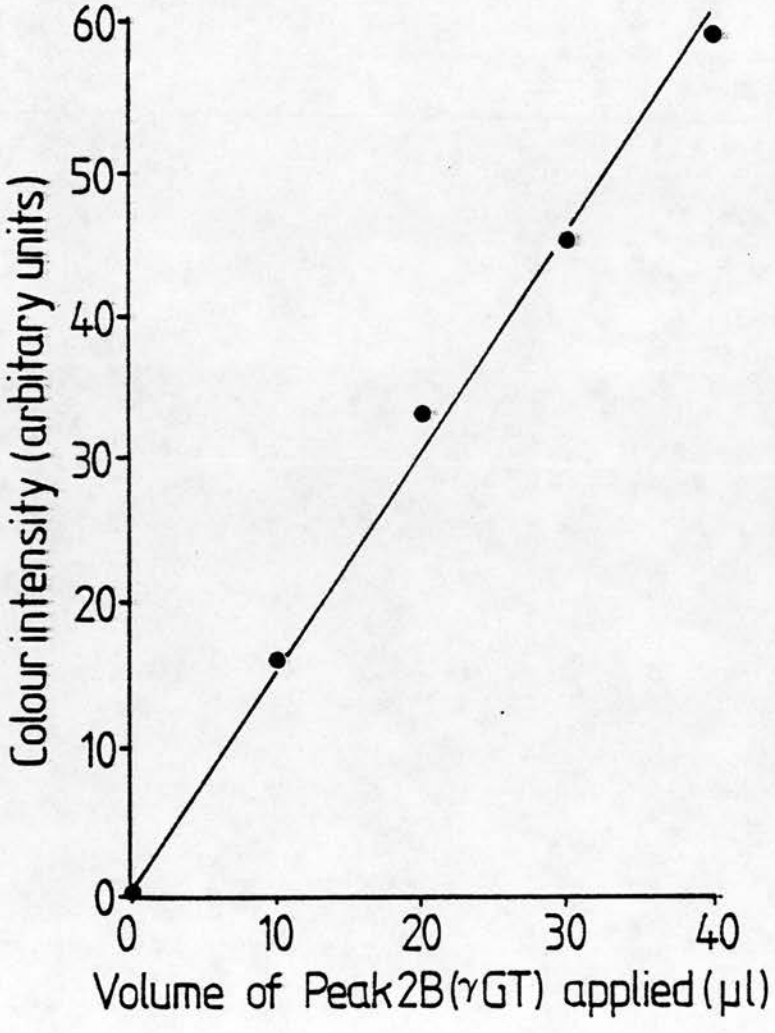


Figure 5.2 Electrophoresis of Peak 2B (γGT) on polyacrylamide gradient gel. Variation in intensity of Band IIB (γGT) with amount of enzyme applied.



5.4 REFERENCE RANGES

Reference ranges for the enzyme activities in serum were as follows:- for γGT, 6 - 31 U/l for females, 8 - 49 U/l for males; for LAP, 27 - 70 U/l and for ALP, 30 - 140 U/l.

5.5 STATISTICAL ANALYSIS

The results of tests in different disease categories were compared using non-parametric methods of analysis (Kruskal-Wallis one-way analysis of variance), (Siegel 1956). Correlation analysis was performed using standard parametric methods.

5.6 HIGH M_r γGT

The distributions of total γGT, high M_r γGT (U/l) and high M_r γGT (%) in the different patient groups are shown in Figs. 5.3 - 5.5. The results from patients with metastatic liver disease are similar, whether or not jaundice was evident ($P = 0.13$), so these two groups have been considered together. In all cases there is considerable overlap between the groups. Nevertheless it is clear that high M_r γGT, expressed as a percentage:-

1. Was greatest in patients with extrahepatic obstruction ($p < 0.000001$).

2. Was greater in patients with liver metastases than in other groups, if the patients with extrahepatic obstruction are excluded from the analysis ($p < 0.0001$).

3. Was greater in patients with extrahepatic obstruction than in those with metastatic liver disease ($p < 0.00001$).

4. Was greater in patients with extrahepatic obstruction than in jaundiced patients in other disease categories ($p < 0.00001$).

Figure 5.3 Distribution of total γGT activity in the different patient groups. —— , arithmetic mean.

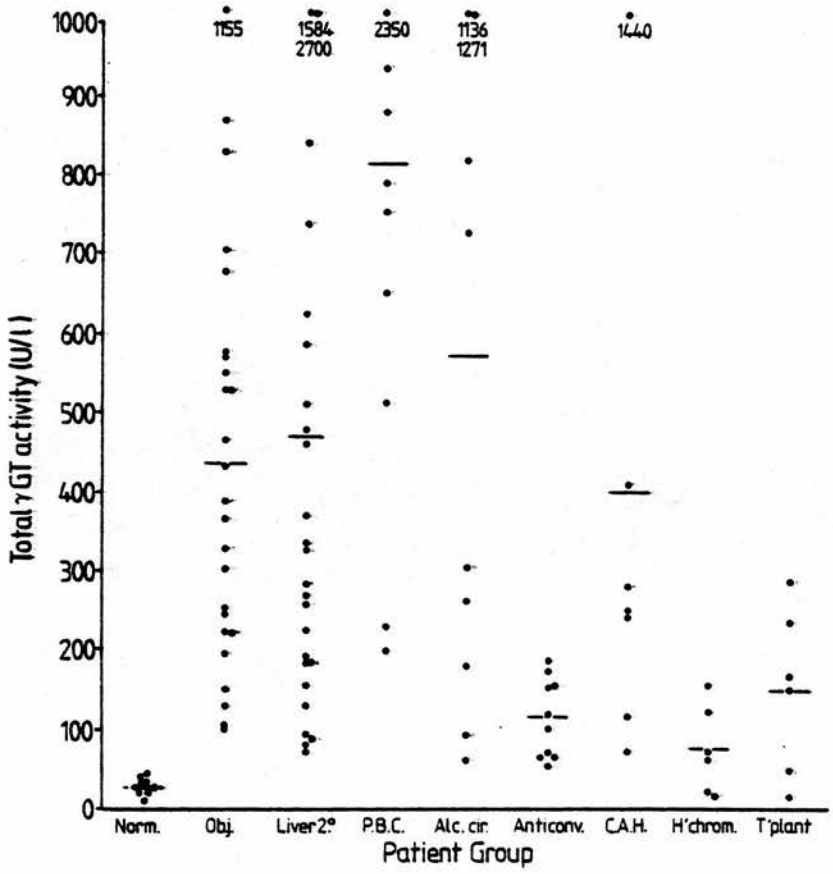


Figure 5.4 Distribution of high $\underline{M}_r$ γGT expressed as U/l in the different patient groups. —— , arithmetic mean.

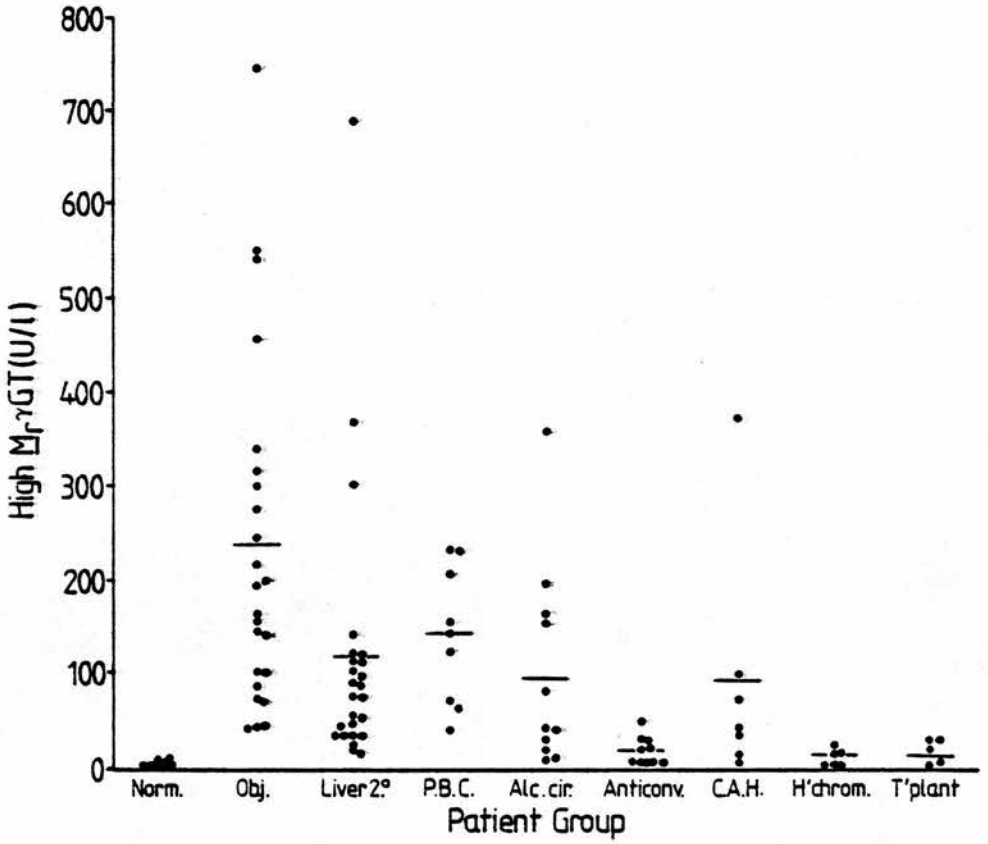
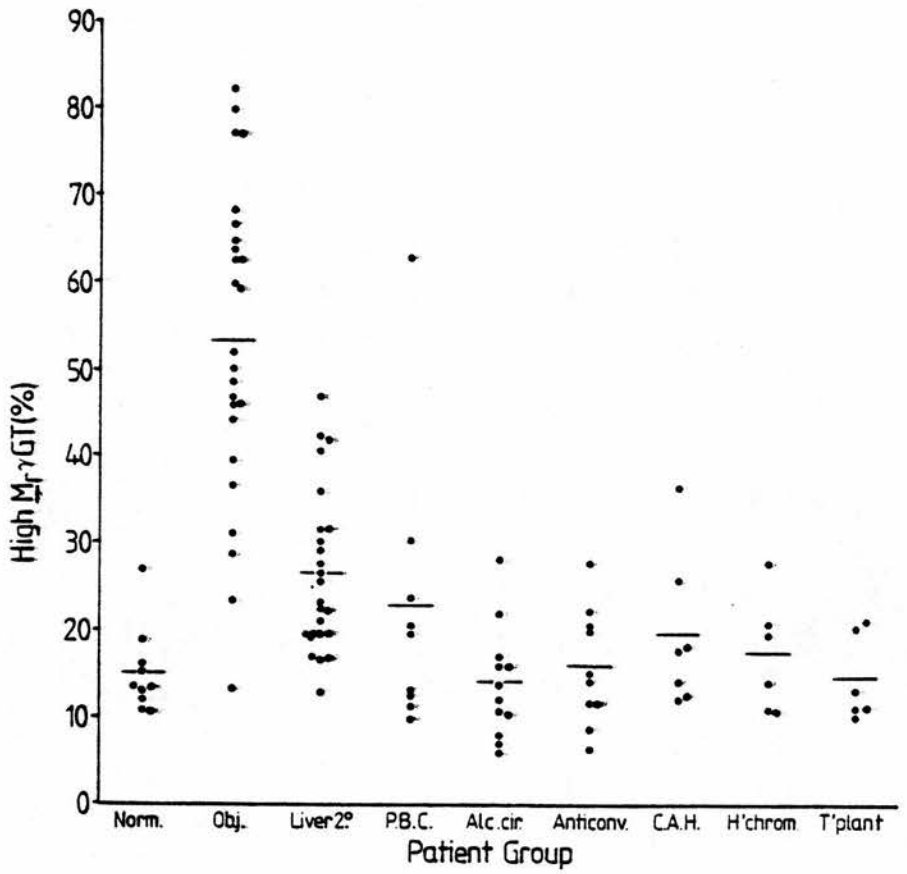


Figure 5.5 Distribution of high M_r γGT expressed as the percentage of the total activity in the different patient groups. —— , arithmetic mean.



High $\underline{M}_r$ γGT (expressed as U/l) tends to be within the reference range or only slightly elevated in those patients receiving anticonvulsants, suffering from haemochromatosis, or after liver transplantation.

5.7 HIGH $\underline{M}_r$ LAP AND HIGH $\underline{M}_r$ ALP

The results for high $\underline{M}_r$ LAP (Figs. 5.6 - 5.8) were very similar to those for high $\underline{M}_r$ γGT in patients with extrahepatic obstruction and metastatic liver disease.

The results for high $\underline{M}_r$ ALP (Figs. 5.9 - 5.11) were also similar to those for high $\underline{M}_r$ γGT, but showed fewer differences between the different disease categories. For example, whereas high Mr ALP (%) was greatest in extrahepatic obstruction ($p < 0.001$), direct comparison between the patients with extrahepatic obstruction and those with liver secondaries or those with jaundice from other causes showed no significant difference. The results of the statistical analysis of the data for the high $\underline{M}_r$ enzymes are shown in Table 5.2.

Figure 5.6 Distribution of total LAP activity in the different patient groups. —— , arithmetic mean.

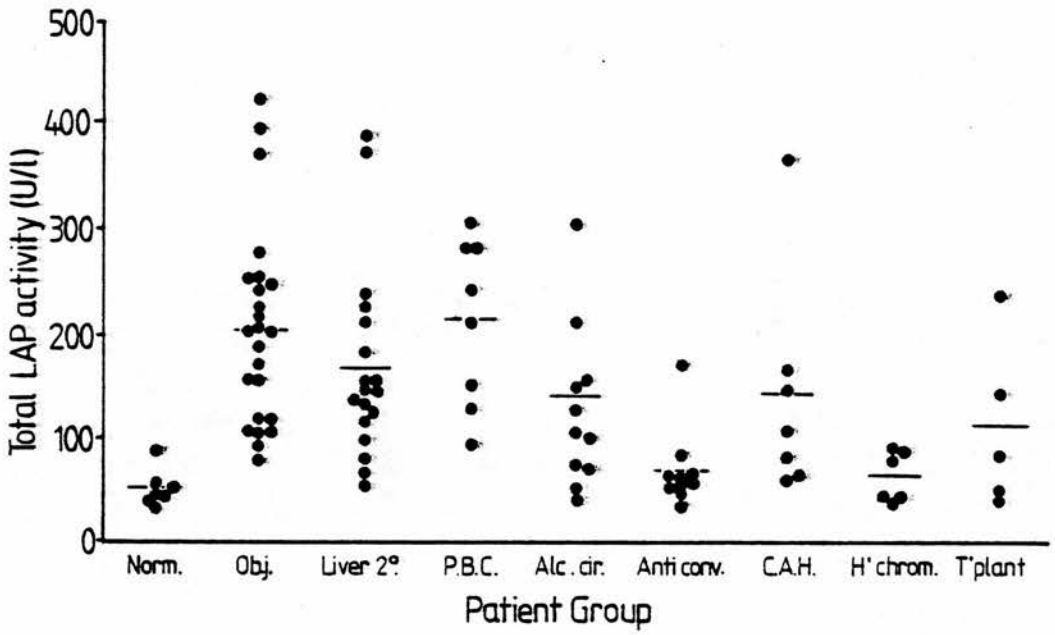


Figure 5.7 Distribution of high M_r LAP expressed as U/l in the different patient groups. —— , arithmetic mean.

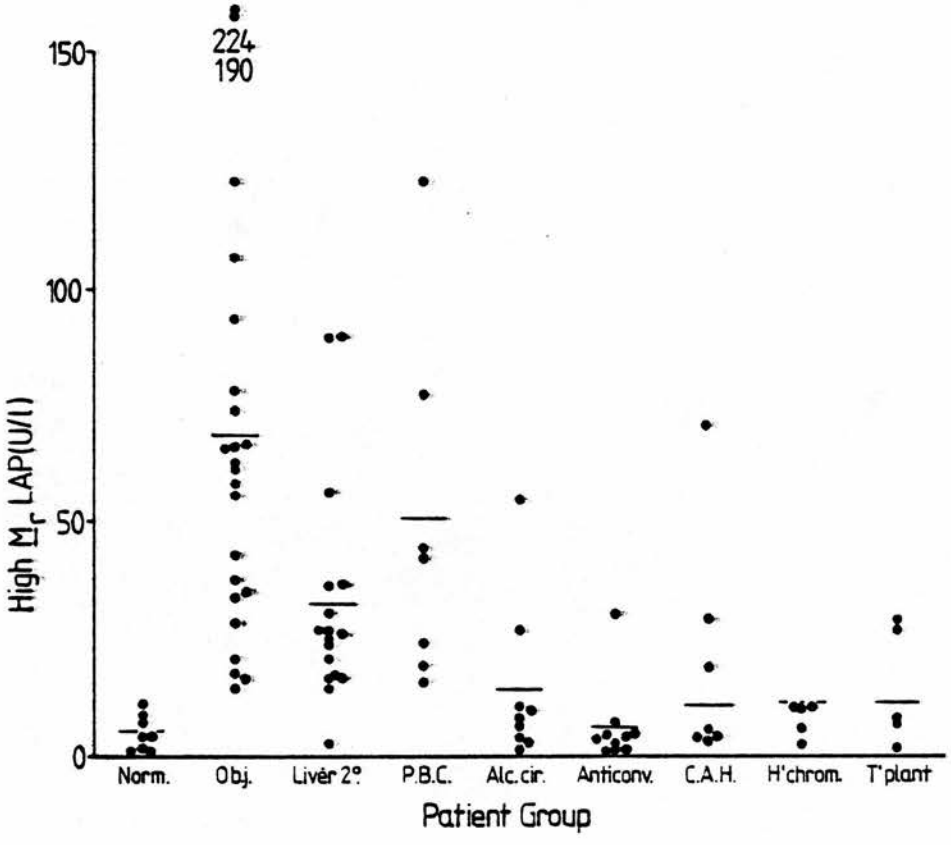


Figure 5.8 Distribution of high $\underline{M}_r$ LAP (%) in the different patient groups. —— , arithmetic mean.

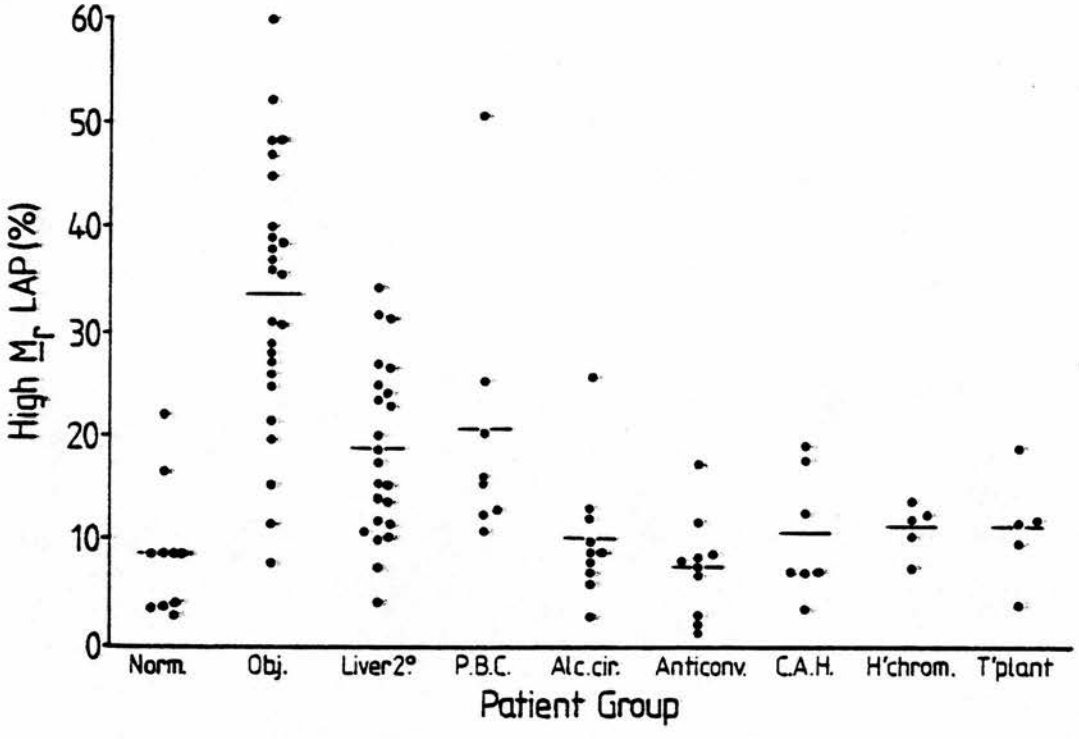


Figure 5.9 Distribution of total ALP activity in the different patient groups. —— , arithmetic mean.

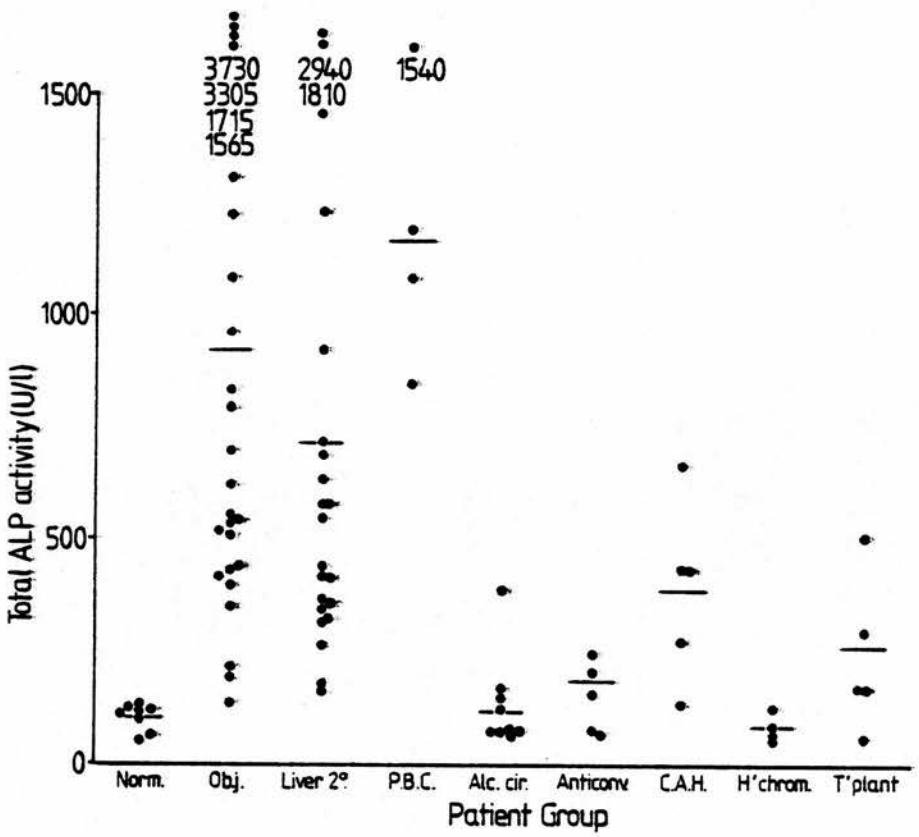


Figure 5.10 Distribution of high M_r ALP expressed as U/l in the different patient groups. —— , arithmetic mean.

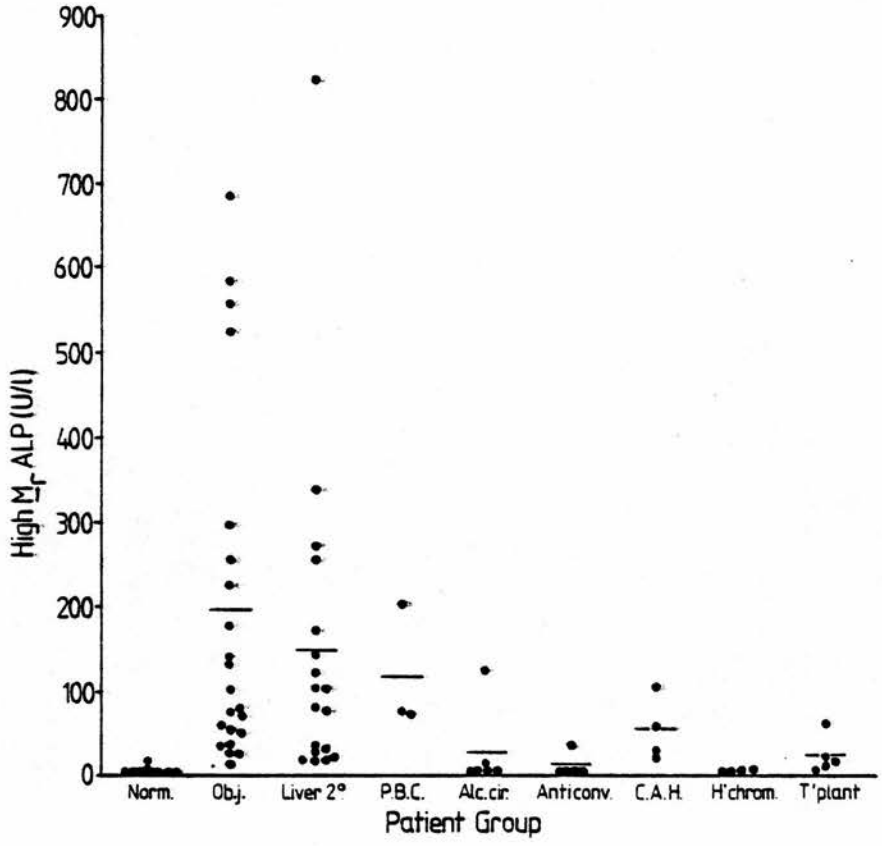


Figure 5.11 Distribution of high M_r ALP expressed as the percentage of total activity in the different patient groups. —— , arithmetic mean.

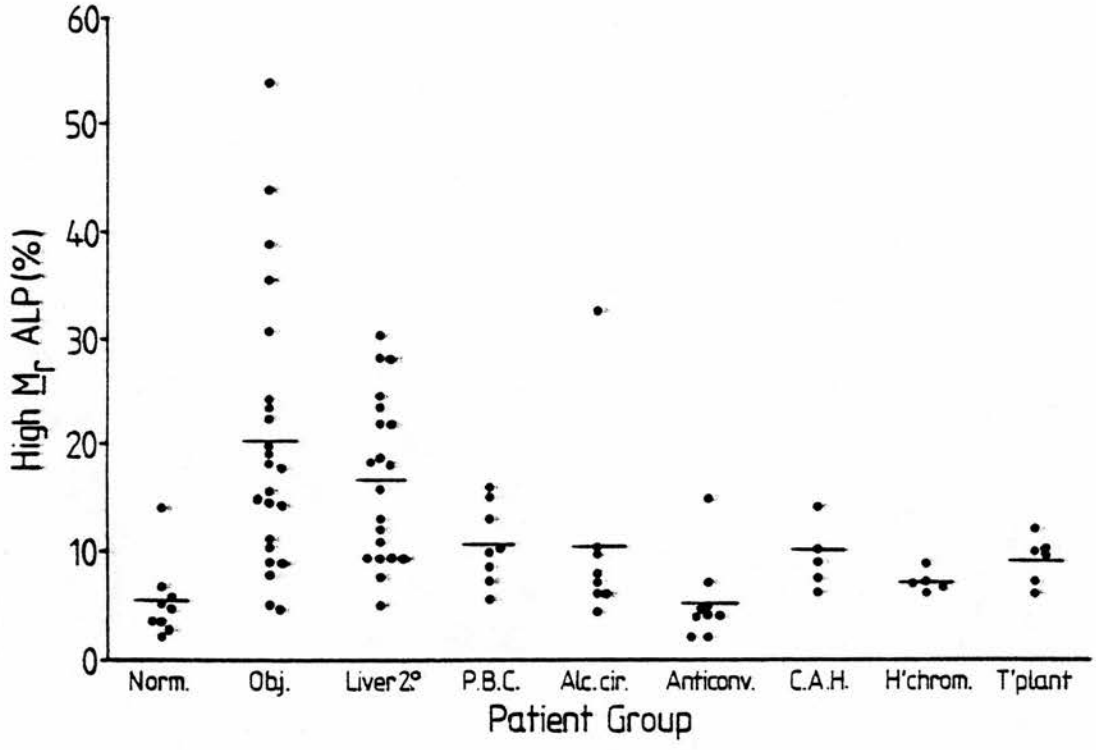


Table 5.2

Result between patient groups of the comparison of high M_r γGT (%), high M_r LAP (%) and high M_r ALP (%)

Patient Groups	γGT	LAP	ALP
Ob.j. (M_1) v All other (M_2)	53 18	34 13	20 10
P[a]	<0.0000001	<0.0000001	<0.001
Ob.j. (M_1) v Liver 2° (M_3)	53 27	34 19	20 18
P[a]	<0.00001	<0.0001	N.S.[b]
Ob.j. (M_1) v All jaundiced sera (M_4)	53 24	34 16	20 14
P[a]	<0.00001	<0.00001	N.S.[b]
Liver 2° (M_3) v All patients minus Ob.j. (M_5)	27 19	19 13	18 9
P[a]	<0.0001	<0.001	<0.0001

[a] P is the probability that the differences between the groups is due to chance alone.

[b] Not significant

M_1 = mean value in patients with obstructive jaundice
M_2 = mean value in all other patients
M_3 = mean value in patients with metastatic liver disease
M_4 = mean value in all jaundiced sera
M_5 = mean value in all patients without obstructive jaundice

5.8 RELATIONSHIP BETWEEN HIGH M_r γGT AND OTHER HIGH M_r ENZYMES

There was a close relationship between high M_r γGT(%) and high M_r LAP (%), r = 0.84 (Fig. 5.12). There was a poorer correlation between high M_r ALP (%) and high γGT (%), r = 0.66 (Fig. 5.13) or high M_r LAP %, r = 0.67 (Fig. 5.14).

Figure 5.12 Relationship between high $\underline{M}_r$ γGT and high $\underline{M}_r$ LAP, both expressed as the percentage of total activity.

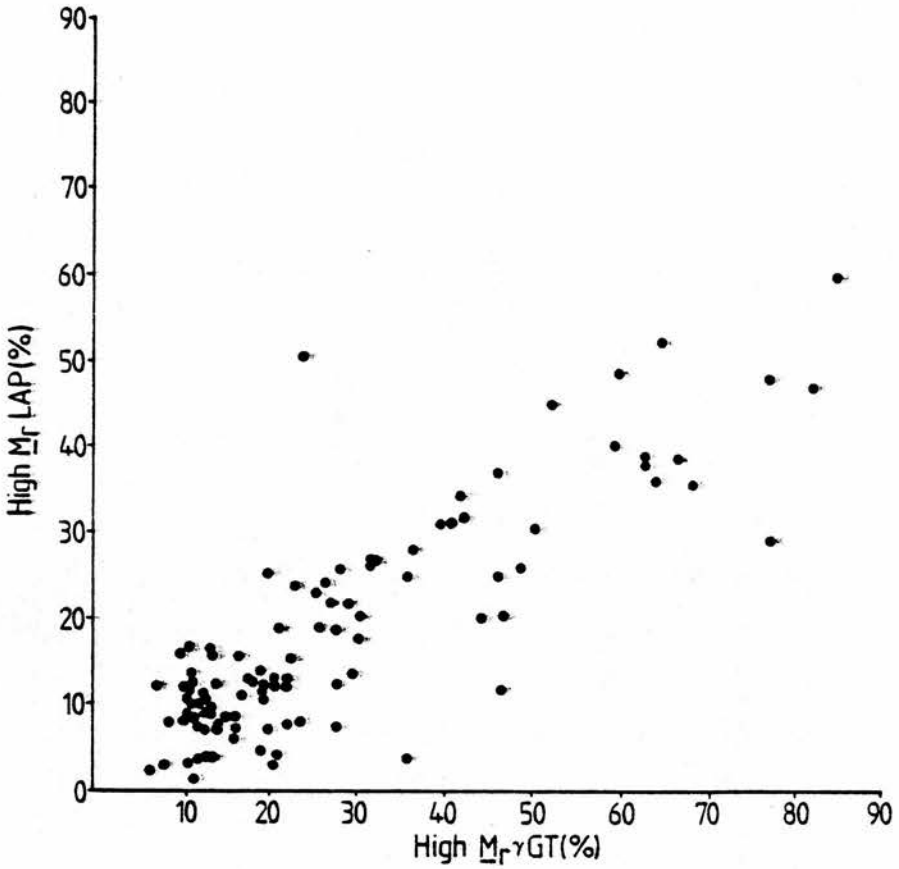


Figure 5.13 Relationship between high $\underline{M}_r$ γGT and high $\underline{M}_r$ ALP, both expressed as the percentage of total activity.

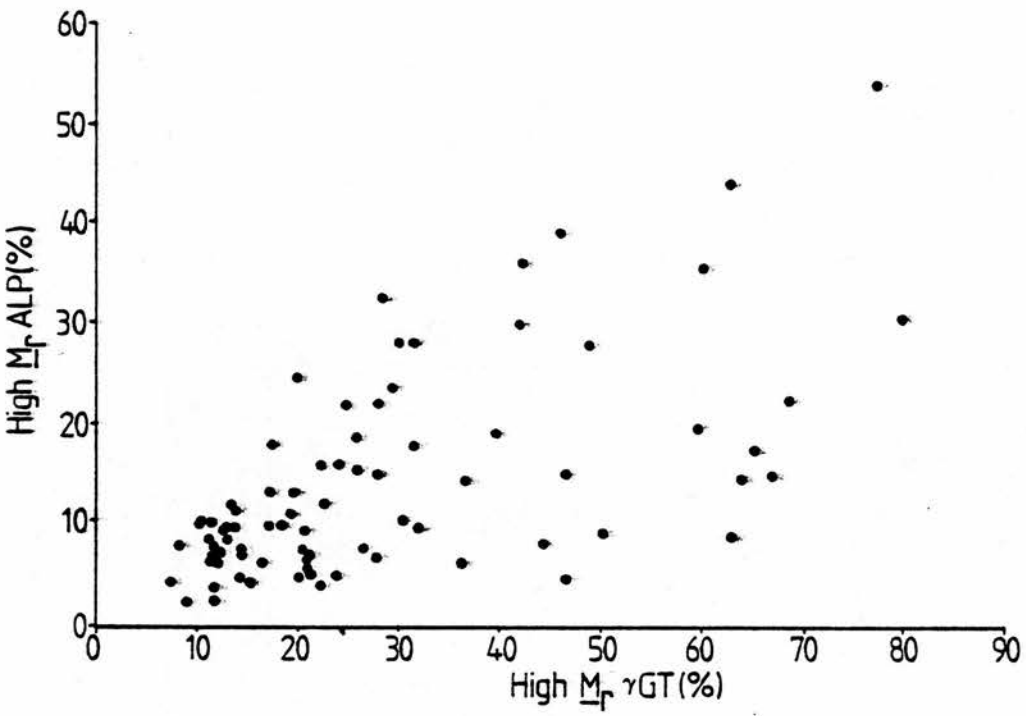
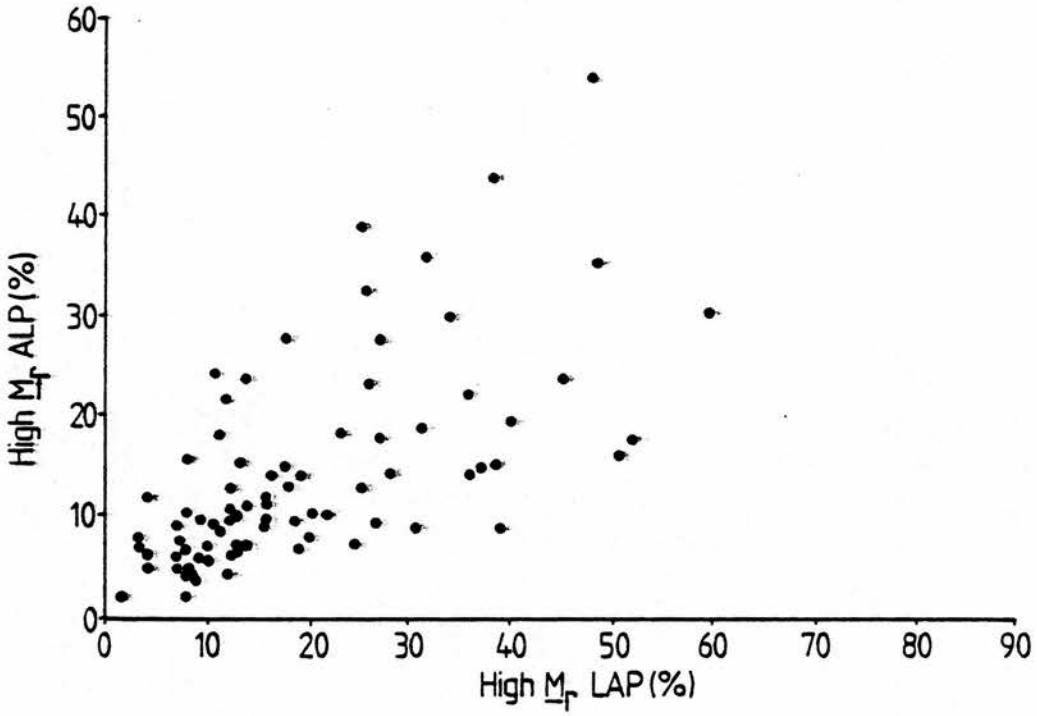


Figure 5.14 Relationship between high $\underline{M}_r$ LAP and high $\underline{M}_r$ ALP, both expressed as the percentage of total activity.



One of the points on the γGT v LAP correlation scattergram (Fig. 5.12), which was from a patient with primary biliary cirrhosis, appears to be obviously discrepant. There were values of 23.9% and 50.5% for high $\underline{M}_r$ γGT and high $\underline{M}_r$ LAP respectively. However, the calculation was confirmed to be correct and nothing else unusual was found about the patient.

Similarly, inspection of the γGT v ALP correlation scattergram (Fig. 5.13) revealed 9 patients with a low value for high $\underline{M}_r$ ALP (%) relative to high $\underline{M}_r$ γGT (%), i.e. all of the high $\underline{M}_r$ ALP (%) values were less than 20%, and all of the high $\underline{M}_r$ γGT (%) values were greater than 43%. Although all of these patients had extrahepatic obstructive jaundice, it was difficult to place any great significance on this observation, due to the large scatter on the data points.

5.9 RELATIONSHIP BETWEEN HIGH $\underline{M}_r$ ENZYMES AND BILE SALT CONCENTRATIONS

Since serum bile salt concentrations are widely regarded as good indices of cholestasis, the relationship between them and the high $\underline{M}_r$ enzymes, measured as percentages of total activity, was investigated. Bile salt concentrations were significantly higher in extrahepatic obstruction than in other forms of liver disease (Table 5.3, $p < 0.000001$). The relationship between the bile salt concentrations and the high $\underline{M}_r$ enzymes is shown in Figs. 5.15 - 5.20). There is a low but significant association between serum bile salt concentrations and high $\underline{M}_r$ γGT and high $\underline{M}_r$ LAP but there is no correlation with high $\underline{M}_r$ ALP (Table 5.4).

Table 5.3

Bile salt concentrations in patients with extrahepatic biliary obstruction compared to all other patients

Patient Group	Total conjugated cholate (μmol/l)		Total conjugated chenodeoxycholate (μmol/l)	
	Mean	Range	Mean	Range
Ob.j.	101	12 - 296	64	9 - 229
All other patients	24	0 - 234	12	0 - 113

Table 5.4

Correlation coefficients between the high M_r enzymes and total conjugated bile salt concentrations

High M_r enzyme	Total conjugated cholate	Total conjugated chenodeoxycholate
	r	r
High M_r γGT (%)	0.47 [a]	0.32 [b]
High M_r LAP (%)	0.38 [a]	0.24 [c]
High M_r ALP (%)	0.065 [d]	0.097 [d]

[a] $P < 0.001$

[b] $P < 0.01$

[c] $P < 0.05$

[d] Not significant

Figure 5.15 Relationship between high $\underline{M}_r$ γGT, expressed as the percentage of total activity and total conjugated cholate.

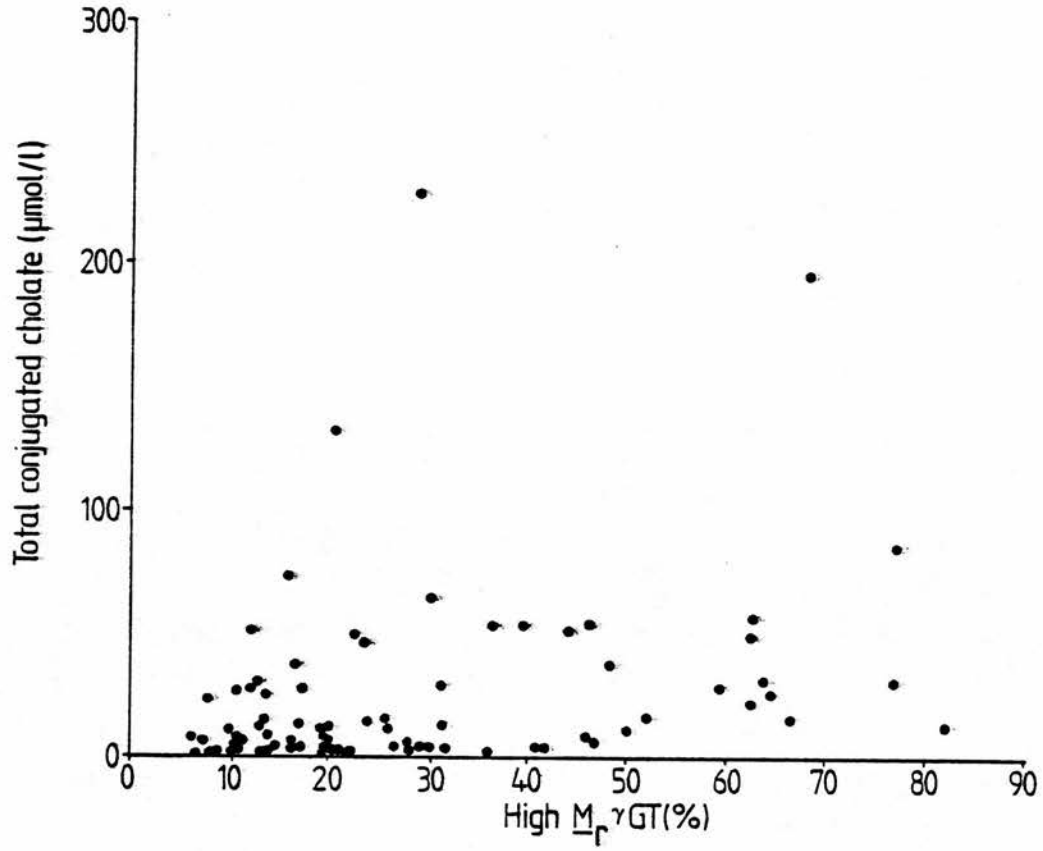


Figure 5.16 Relationship between high $\underline{M}_r$ γGT, expressed as the percentage of total activity, and total conjugated chenodeoxycholate.

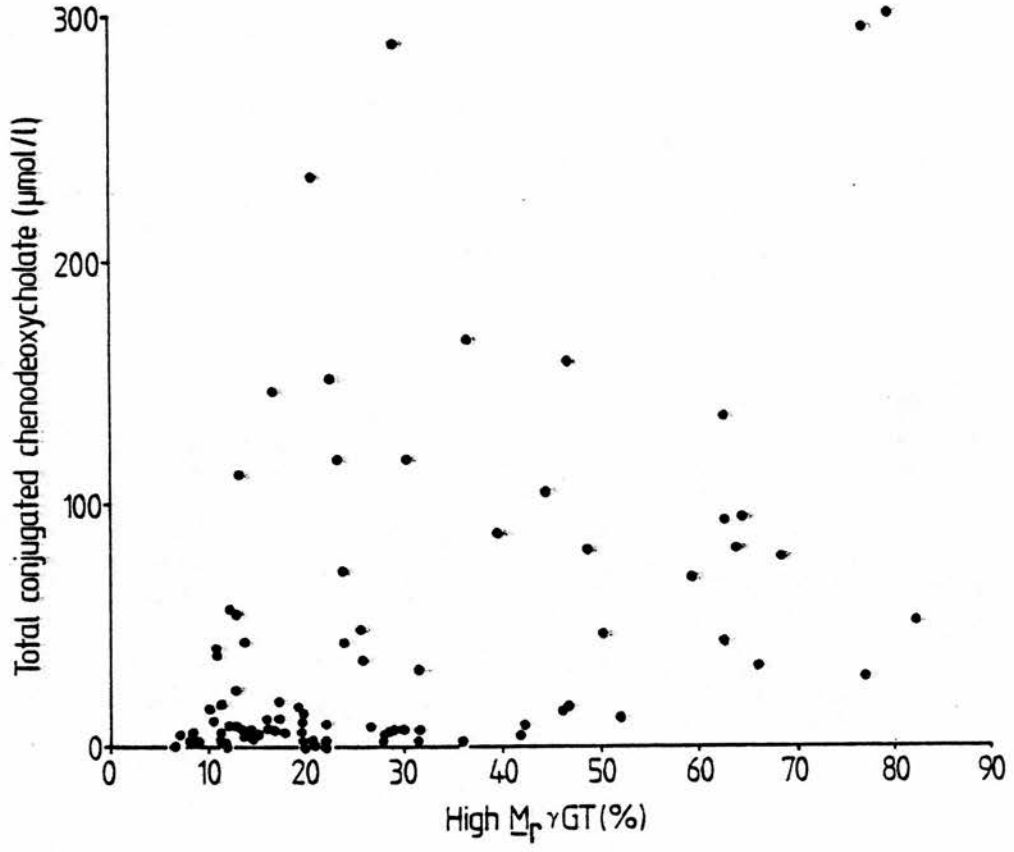


Figure 5.17 Relationship between total conjugated cholate and high $\underline{M}_r$ LAP, expressed as the percentage of total activity.

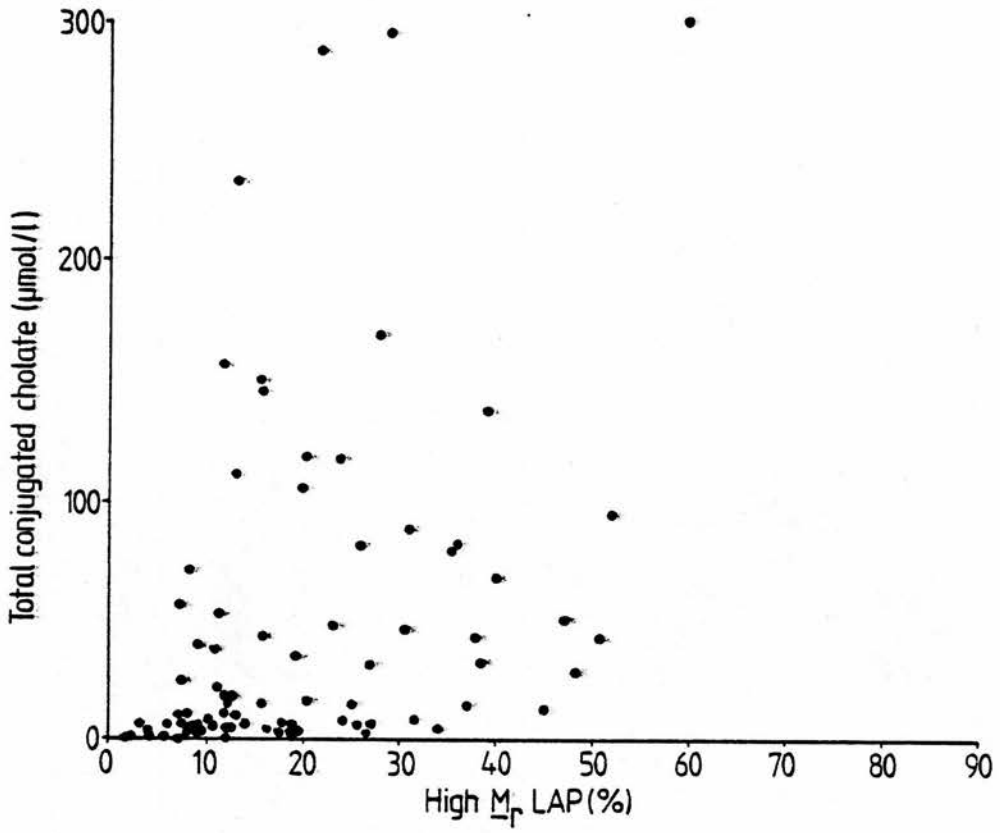


Figure 5.18 Relationship between high $\underline{M}_r$ LAP, expressed as the percentage of total activity, and total conjugated chenodeoxycholate concentration.

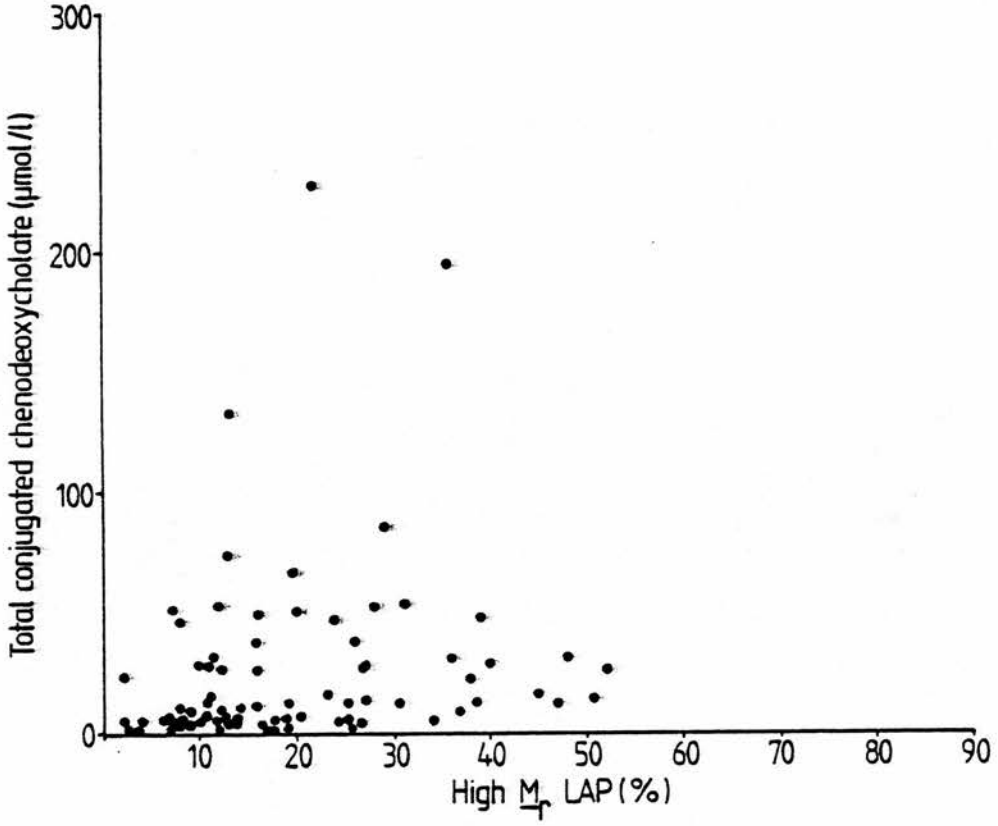


Figure 5.19 Relationship between high $\underline{M}_r$ ALP, expressed as the percentage of total activity, and total conjugated cholate.

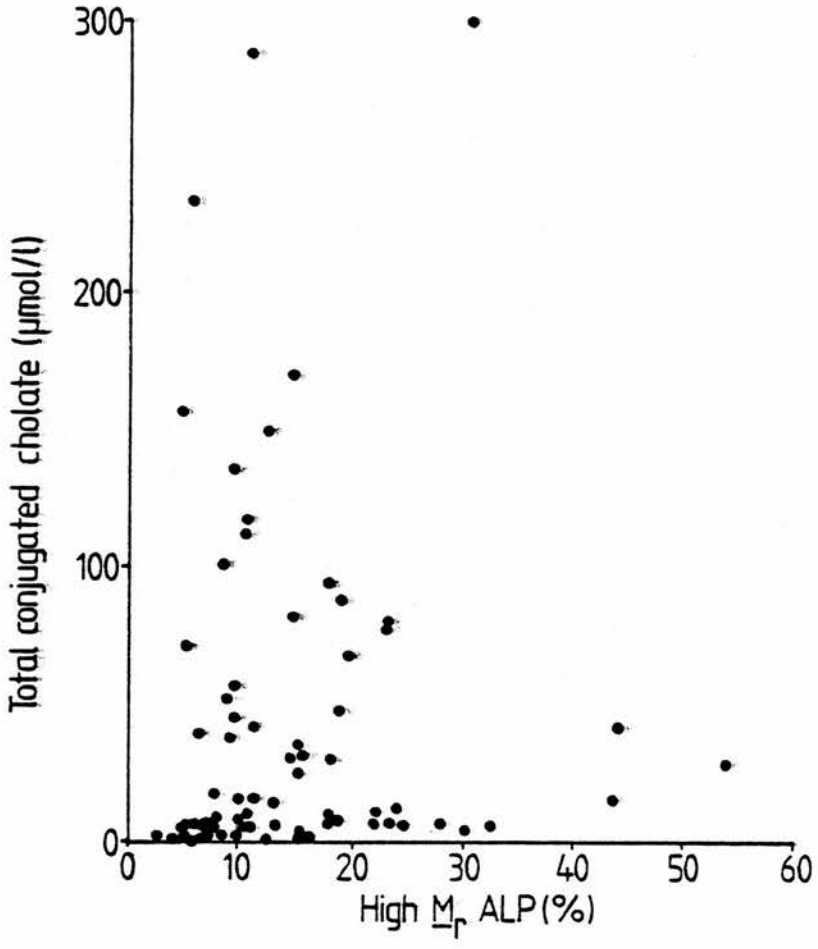
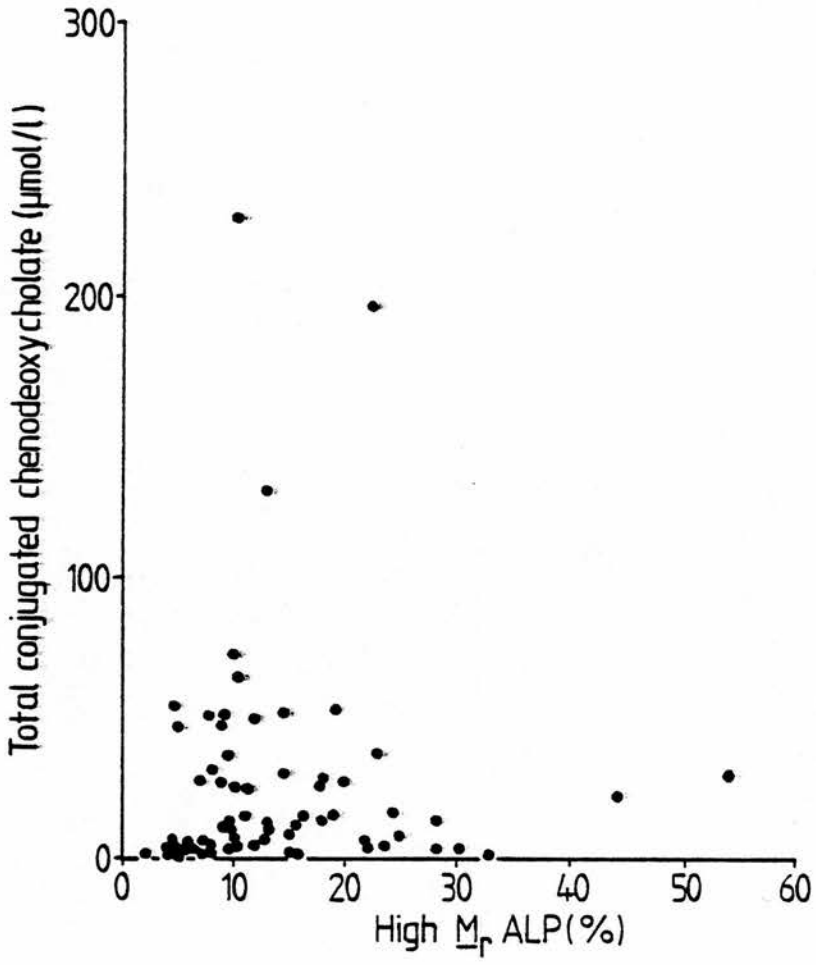


Figure 5.20 Relationship between high $\underline{M}_r$ ALP, expressed as the percentage of total activity, and total conjugated glycochenodeoxycholate.



5.10 QUANTITATION OF BAND IIB (γGT)

Quantitative measurement of Band IIB (γGT) was made in 64 of the patients with liver disease, including 16 of the 25 patients with jaundice due to extrahepatic obstruction. Typical electrophoretic patterns are shown in Fig. 5.21 and the results plotted in relation to high $\underline{M}_r$ γGT (%) in Fig. 5.22. In the occasional cases where Bands IIA and IIB (γGT) were present together in the serum and they were not completely separated, e.g. patients 5 and 8 (Fig. 5.21), the percentages were calculated by extrapolation of the individual peaks. In 11 of the 16 patients (69%) with obstructive jaundice, all of the intermediate $\underline{M}_r$ γGT was present as Band IIB (γGT) as seen by visual inspection of the gels and confirmed by densitometric scanning. In none of the patients from any of the other groups was all of the intermediate $\underline{M}_r$ γGT present as Band IIB (γGT). Although the numbers are quite small, the data in Fig. 5.22 suggest that the electrophoretic subdivision of Band II (γGT) discriminates between the patients with extrahepatic obstruction and the rest better than the measurement of high $\underline{M}_r$ γGT (%). In an attempt to determine whether the two results combined further improved discrimination, Fig. 5.22 was arbitrarily divided into 4 quadrants using cut-off values derived from the value of the mean +2 S.D. for each variable in the patients without obstructive jaundice (40% for high $\underline{M}_r$ γGT, 57% for Band IIB (γGT)). A positive test for obstructive jaundice was defined as a value in the top right quadrant (i.e. both variables above the cut-off limit). The results obtained, compared to those derived from using only one of the variables (with the same cut-off limits) are shown in Table 5.5 together with equivalent results obtained by measuring high $\underline{M}_r$ LAP (%) (cut-off value 32%) and high $\underline{M}_r$ ALP (%) (cut-off value 26%). They suggest that little improvement in discrimination is obtained by measuring high $\underline{M}_r$ γGT (%) and Band IIB (γGT) together as compared with that obtained from electrophoretic fractionation alone. The results also show that, whereas all three high $\underline{M}_r$ enzymes had similar values for specificity in the detection of extrahepatic biliary obstruction, high $\underline{M}_r$ ALP (%) was much less sensitive than either high $\underline{M}_r$ γGT (%) or high $\underline{M}_r$ LAP %.

Figure 5.21 Polyacrylamide gradient gel electrophoresis of sera from patients with liver disease. 1 - 4, extraheptic biliary obstruction; 5 - 9, alcoholic cirrhosis.

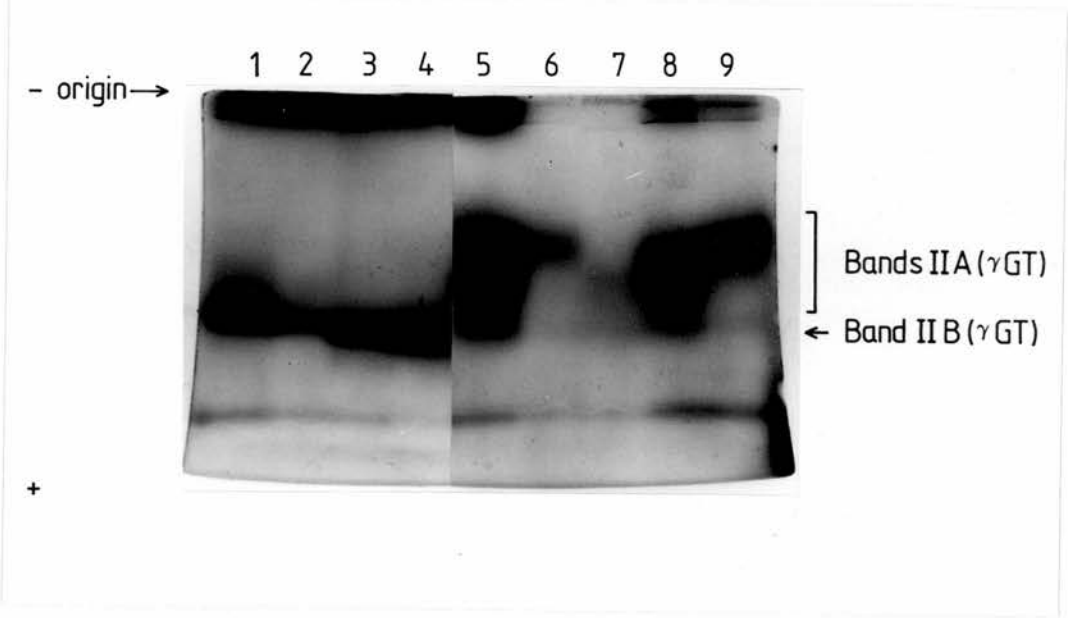


Figure 5.22 Band IIB (γGT), expressed as the percentage of all intermediate $\underline{M}_r$ γGT, plotted in relation to high $\underline{M}_r$ γGT, expressed as a percentage of the total γGT activity, in patients with liver disease. ● , extrahepatic biliary obstruction; ○ , others. See section 5.10 for the meaning of the lines.

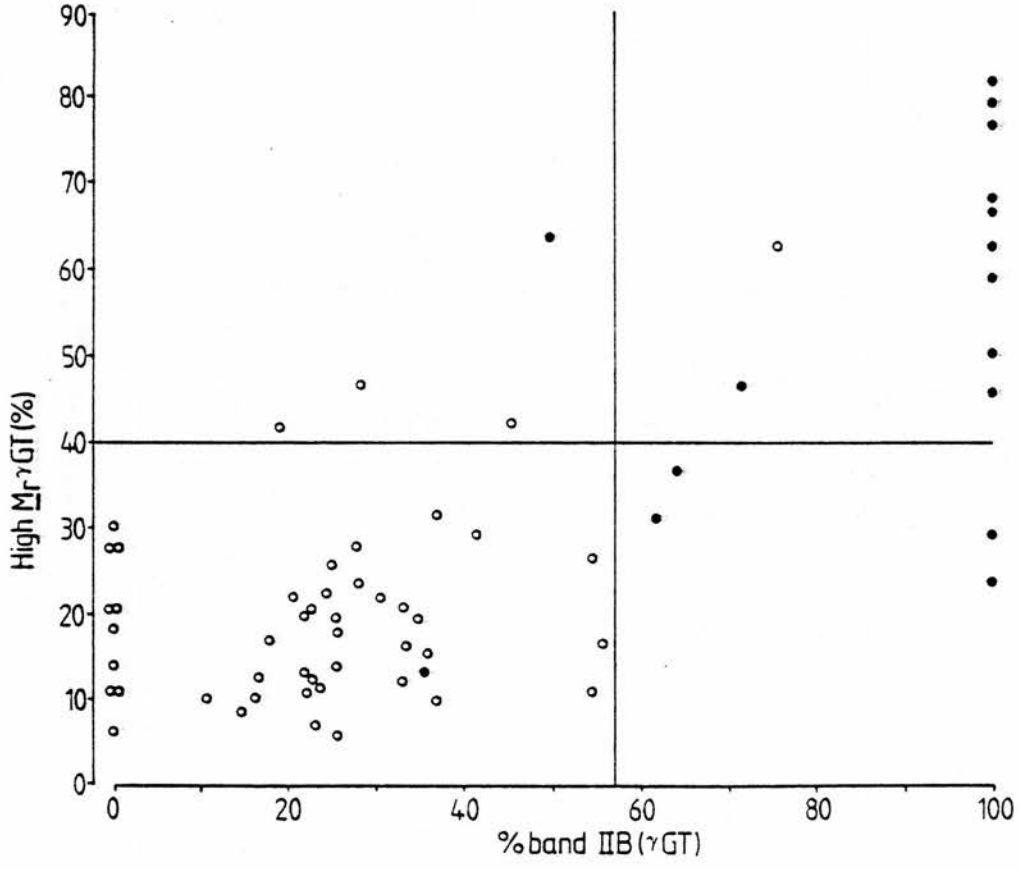


Table 5.5

Diagnostic value of high M_r γGT (%) and % Band IIB (γGT) measured singly or together, high M_r LAP (%) and high M_r ALP (%), in the detection of extrahepatic biliary obstruction

	Number (and %) of total patients				
	High M_r γGT	Band IIB	Both	High M_r LAP	High M_r ALP
Sensitivity[a]	11/16 (69)	14/16 (88)	10/16 (63)	9/16 (56)	3/15 (20)
Specificity[b]	44/48 (92)	46/48 (96)	47/48 (98)	40/42 (95)	38/40 (95)
Predictive value of positive test[c]	11/15 (73)	14/16 (88)	10/11 (91)	9/11 (82)	3/6 (50)
Predictive value of negative test[d]	44/49 (90)	46/48 (96)	47/53 (89)	40/47 (85)	35/47 (74)

[a] Proportion of patients with extrahepatic obstruction giving a positive test.

[b] Proportion of patients without extrahepatic obstruction giving a negative test.

[c] Proportion of positive tests with extrahepatic obstruction.

[d] Proportion of negative tests without extrahepatic obstruction.

5.11 DISCUSSION

In this chapter, the discussion is limited to the relationship of the results to diagnosis. Discussion of the results in the light of possible mechanisms for the appearance of the enzymes in the circulation will be considered in the final chapter (under the heading 'General Discussion').

The results confirm many previous reports that serum γGT activity is elevated in most types of liver disease. In the present study an attempt was made to increase its discriminatory capacity by performing more complex analysis of serum γGT patterns in liver disease.

Despite the obvious constraints imposed upon the study by time and limitations in patient numbers, the investigation has shown that high $\underline{M}_r$ γGT, particularly when the results are expressed as a percentage, is (1) higher in obstructive than non-obstructive lesions, and (2) higher in extrahepatic than in intrahepatic obstruction. These results suggest that measurement of high $\underline{M}_r$ γGT, particularly when the results are expressed as a percentage, can help to distinguish extrahepatic (a) from intrahepatic obstruction and (b) from almost every other form of liver disease, whether or not the patient is jaundiced. Moreover, a high value (greater than 50%) appears to be almost diagnostic of extrahepatic obstructive jaundice (Fig. 5.5).

Only one systematic clinical study of high $\underline{M}_r$ γGT in patients with liver disease appears to have been made (Moss *et al.*, 1982). These authors found significantly higher values in 38 patients with malignant infiltration of the liver than in 17 patients with alcoholic liver disease. In both groups, however, the mean total serum γGT activities were similar. These results have been confirmed in the present study although considerable overlap occurred between these particular groups. More recently, Selvaraj *et al.* (1984), in a study of total hydrophobic γGT, were unable to distinguish between different types of liver disease. However, their method, which involved relatively non-specific binding of hydrophobic γGT to phenyl-Sepharose, would also measure intermediate $\underline{M}_r$ γGT, as well as high $\underline{M}_r$ γGT.

High $\underline{M}_r$ LAP behaved similarly to high $\underline{M}_r$ γGT and was also present in greater amounts in obstructive than in non-obstructive lesions.

In notable contrast to high $\underline{M}_r$ γGT and high $\underline{M}_r$ LAP, several clinical studies involving the measurement of high $\underline{M}_r$ ALP have been made (Fennelly et al., 1969; Price and Sammons, 1976; Crofton et al., 1979; Siede and Sieffert, 1983). These studies have also found higher levels in obstructive than in non-obstructive lesions. There is, however, some disagreement as to whether the highest levels are observed in extrahepatic obstruction or metastatic liver disease (Fennelly et al., 1969; Price and Sammons, 1976; Crofton et al., 1979). Although the present investigation found that highest levels occurred in extrahepatic obstruction (Fig. 5.11), there was no significant difference between these and the values seen in patients with liver metastases (Table 5.2).

The measurement of high $\underline{M}_r$ ALP has been advocated as having particular value in the detection of metastatic liver disease (Viot et al., 1981; Karmen et al., 1984). The present study confirms that higher levels are present in patients with secondary deposits compared to patients with liver disease other than extrahepatic biliary obstruction. However, these results do not suggest that the measurement of high $\underline{M}_r$ fractions of γGT, or of LAP and ALP, is likely to be of practical value in the diagnosis and detection of metastatic liver disease, since the overlap with other categories of liver disease is so great. One group of patients however was omitted from the study, namely those with minimal abnormalities of liver function - a group in which the detection of metastatic liver disease might be important. Such a group however was studied by Moss et al. (1982) who concluded that measurement of high $\underline{M}_r$ γGT was no more sensitive in detecting such metastases than the measurement of total serum γGT activity or total, or liver specific, alkaline phosphatase.

The results do suggest that the measurement of Band IIB (γGT), as a percentage of total intermediate $\underline{M}_r$ γGT, particularly when its value is 100%, may distinguish extrahepatic biliary obstruction from other types of liver disease. The technique could prove especially valuable in daily practice if it can be confirmed that visual assessment, without the need for scanning, is adequate for distinguishing most cases.

The measurement of Band IIB (γGT) was combined with that of high $\underline{M}_r$ γGT in an attempt to increase the discrimination between patients who did and did not have extrahepatic obstructive jaundice. This strategy was unsuccessful however (Table 5.5) as no greater discrimination was obtained than by measuring Band IIB (γGT) alone.

In conclusion, the present study has shown that the measurement of high $\underline{M}_r$ γGT or of the intermediate $\underline{M}_r$ form which has been called Band IIB (γGT), may aid in diagnosis in liver disease. High $\underline{M}_r$ γGT may distinguish extrahepatic obstructive causes of jaundice from other causes, but the method is time consuming and tedious rendering it unsuitable for obtaining a result that could be of immediate value to patients. In contrast, estimation of Band IIB (γGT) is relatively simple and is probably a better discriminator. It is considered that electrophoresis of γGT on polyacrylamide gel (or possibly other gels as well) can aid the important clinical differentiation between extrahepatic and intrahepatic causes of jaundice. Moreover, it is sufficiently practical for use in a service laboratory.

Chapter 6

PHYSICAL PROPERTIES OF γ-GLUTAMYLTRANSFERASE IN HUMAN LIVER: RELATIONSHIP TO γ-GLUTAMYLTRANSFERASE IN BILE AND SERUM

γ-Glutamyltransferase in serum probably originates from the liver because increases in serum γGT activity are largely confined to liver disease (Huseby, 1981). The results of the experiments described in Chapters 3 and 4 of this thesis have shown that γGT is present in human bile and serum mainly as hydrophobic forms, together with small amounts of a low $\underline{M}_r$ hydrophilic form (Peak 4 (γGT)). In serum, the hydrophobic γGT is of both high (Peak 1 (γGT)) and intermediate $\underline{M}_r$ (Peak 2 (γGT)), whereas in bile it is of high $\underline{M}_r$ only. In the presence of an adequate concentration of bile salts, the hydrophobic high and intermediate $\underline{M}_r$ forms are dispersed and converted to hydrophobic low $\underline{M}_r$ forms (Peak 3 (γGT)). These low $\underline{M}_r$ forms reaggregate in the absence of bile salts.

In studies attempting to reproduce the electrophoretically separable zones of γGT activity seen in serum, liver tissue was incubated with a normal serum pool and various protein-free solutions (Echetebu and Moss, 1979; Moss, 1980; Echetebu and Moss, 1982a). These authors compared the eluted fractions with those found in a pool of abnormal sera with high γGT activities, in terms of electrophoretic mobility and $\underline{M}_r$. They noted, in particular, that a high $\underline{M}_r$ form, produced by incubation of liver tissue in serum, resembled a form present in the high activity serum pool. They tentatively interpreted this fraction as corresponding to the elution of γGT, in association with components of the membrane matrix, from liver or biliary tract cells.

The preliminary investigation described in this chapter is an attempt to reproduce and extend their findings. Liver tissue has been incubated with abnormal as well as normal serum, protein and protein-free solutions, bile and bile salts, and the eluted enzyme studied. The physical properties of γGT in liver microsomes have also been investigated and a direct comparison made

with the physical properties of γGT in human bile and serum. The aim of the study was to gain further insight into the mechanism behind the findings of the raised serum γGT activity seen in liver disease.

6.1 PHYSICAL PROPERTIES OF γGT IN HUMAN LIVER

6.1.1 Samples used in study

Samples of human liver were obtained post mortem, within 24 h of death, from patients with no evidence or history of liver disease. The liver was washed with ice cold saline, and homogenised in ice cold 20 mmol/l Tris-HCl buffer, pH 8.0, containing 50 mmol/l NaCl (9 g of tissue per 20 ml of buffer). The liver homogenate was centrifuged for 30 min at 3000 x g to remove cell debris and then the supernatant for a further 30 min at 18 000 x g to remove mitochondria. The microsomal fraction was obtained by centrifugation of the supernatant at 100 000 x g for 1 h.

6.1.2 Treatment with sodium deoxycholate

The crude microsomal preparation was incubated with an approximate 10-fold volume of 25 mmol/l sodium deoxycholate for 1 h at room temperature. After centrifugation at 100 000 x g for 1 h, the supernatant was kept for further study.

6.1.3 Treatment with papain

Samples of liver microsomes were incubated with an approximately equal volume of papain (24.5 g/l) and a 10-fold volume of Tris-HCl, pH 8.0, containing 50 mmol/l NaCl overnight at 20°C in the presence of 100 mmol/l cysteine. The samples were then centrifuged for 1 h at 100 000 x g and the supernatant kept for further study.

6.1.4 Gel filtration chromatography

This was carried out on a 2.6 x 65 cm column of Sephadex G200, as described in section 2.3.1. The column was calibrated as described in section 2.3.3 and the same nomenclature used to describe the peaks as used in section 3.4.1.

After gel chromatography of deoxycholate-treated liver microsomes, with increasing concentrations of deoxycholate in the eluting buffer, the main peak of γGT activity changed from Peak 1 (γGT), when there was no deoxycholate in the elution buffer, to peak 3 (γGT) when the deoxycholate concentration was 5 mmol/l or greater (Fig. 6.1). When a concentrated pool of Peak 3 (γGT), (obtained by performing gel chromatography of deoxycholate-treated liver microsomes in the presence of 7 mmol/l deoxycholate), was rechromatographed in the absence of bile salts, the elution profile changed from Peak 3 (γGT) to Peak 1 (γGT).

Gel chromatography of hepatic bile or serum in the presence of at least 5 mmol/l sodium deoxycholate, revealed that the Peak 3 forms of γGT in bile, serum and liver microsomes all had identical elution volumes (Fig. 6.2).

Gel chromatography of the supernatant from the papain-treated microsomes revealed that all of the activity eluted as Peak 4 (γGT). This peak had an identical elution volume to that of the Peak 4 (γGT) obtained following gel chromatography of papain-treated bile (section 3.2) and of papain-treated serum (section 4.2) (Fig. 6.3).

6.1.5 7% polyacrylamide gel electrophoresis

Electrophoresis in 7% polyacrylamide gel, equilibrated in both the gel and the running buffer with at least 5 mmol/l of sodium deoxycholate, showed that deoxycholate-treated liver microsomes contained a main zone of activity (Band III (γGT)) which migrated just ahead of Peak 4 (γGT) (Band IV (γGT)). The Band III (γGT) and Band IV (γGT) obtained from liver microsomes had identical electrophoretic mobilities to that of Band III (γGT) and of Band IV (γGT) obtained from bile (section 3.5.2) and serum (section 4.4.2) (Fig. 6.4).

Figure 6.1 Elution profile on Sephadex G200 of γGT in deoxycholate-treated liver microsomes. There were increasing concentrations of deoxycholate in the eluting buffer. ○ , no deoxycholate; □ , 1.2 mmol/l deoxycholate; ● , 7 mmol/l deoxycholate.

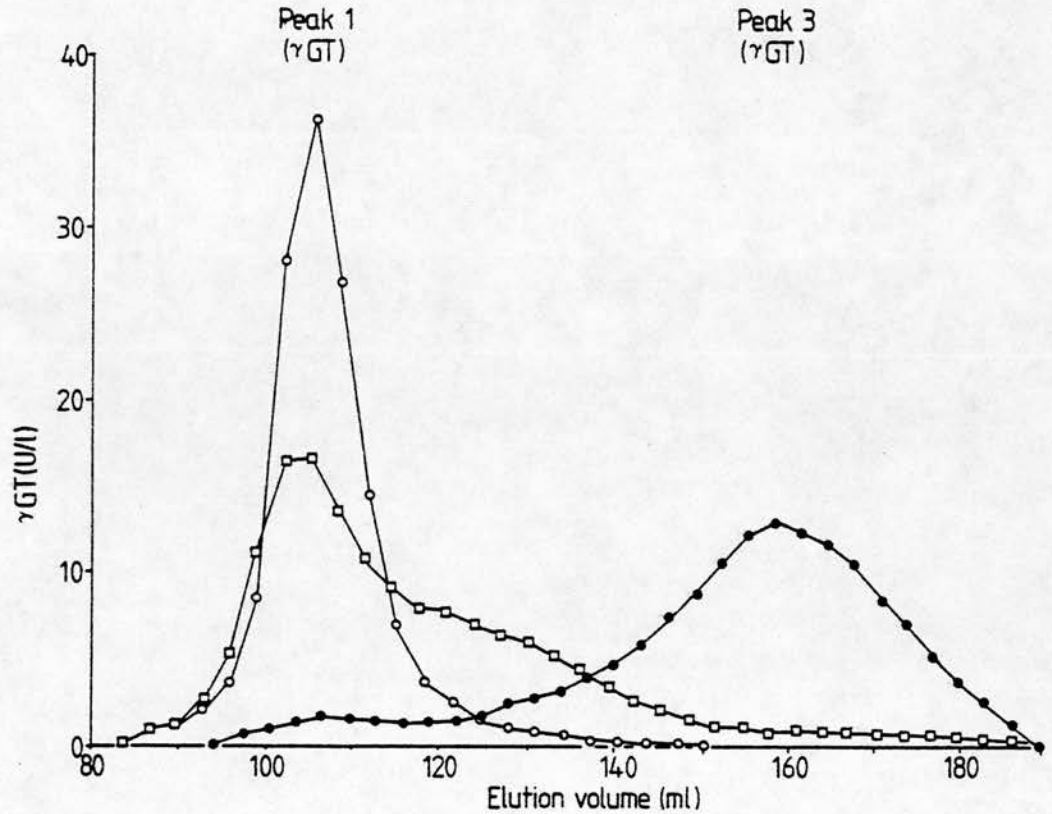


Figure 6.2 Elution profile on Sephadex G200 of γGT in deoxycholate-treated hepatic bile, serum and liver microsomes. The concentration of deoxycholate in the elution buffer was 7.2 mmol/l. ○ , hepatic bile; □ , serum; ● , liver microsomes.

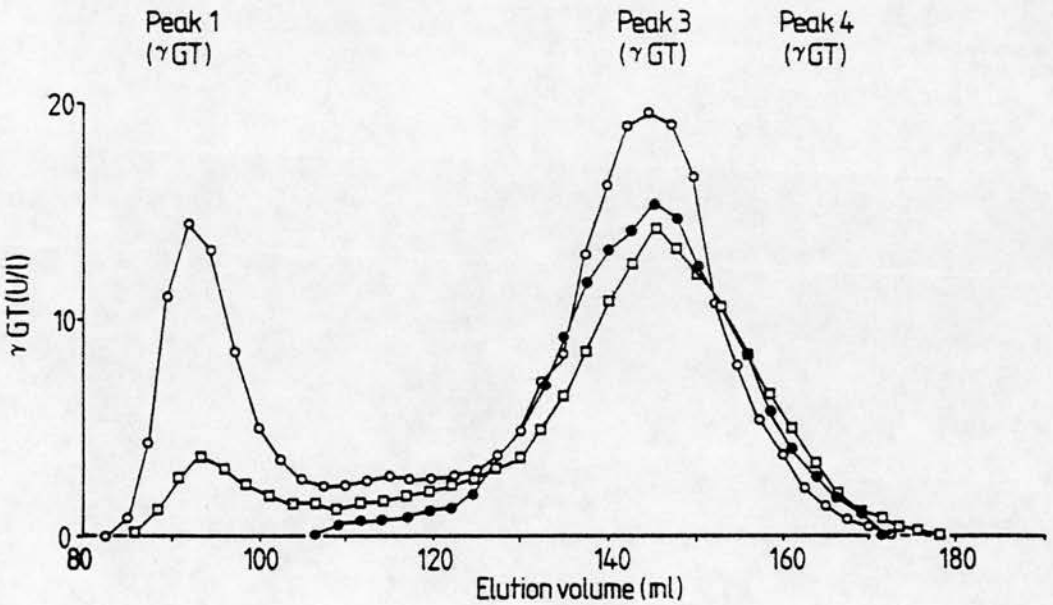


Figure 6.3 Sephadex G200 gel chromatography of papain-treated γGT present in hepatic bile, serum and liver microsomes. ● , hepatic bile; ○ , serum; □ , liver microsomes.

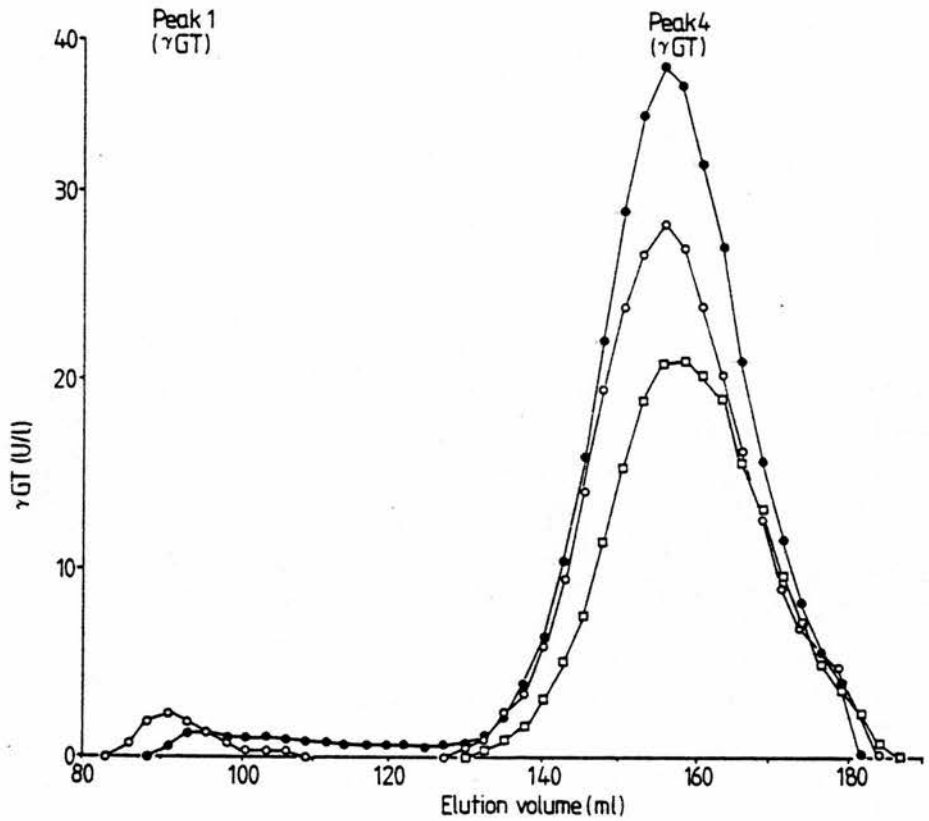
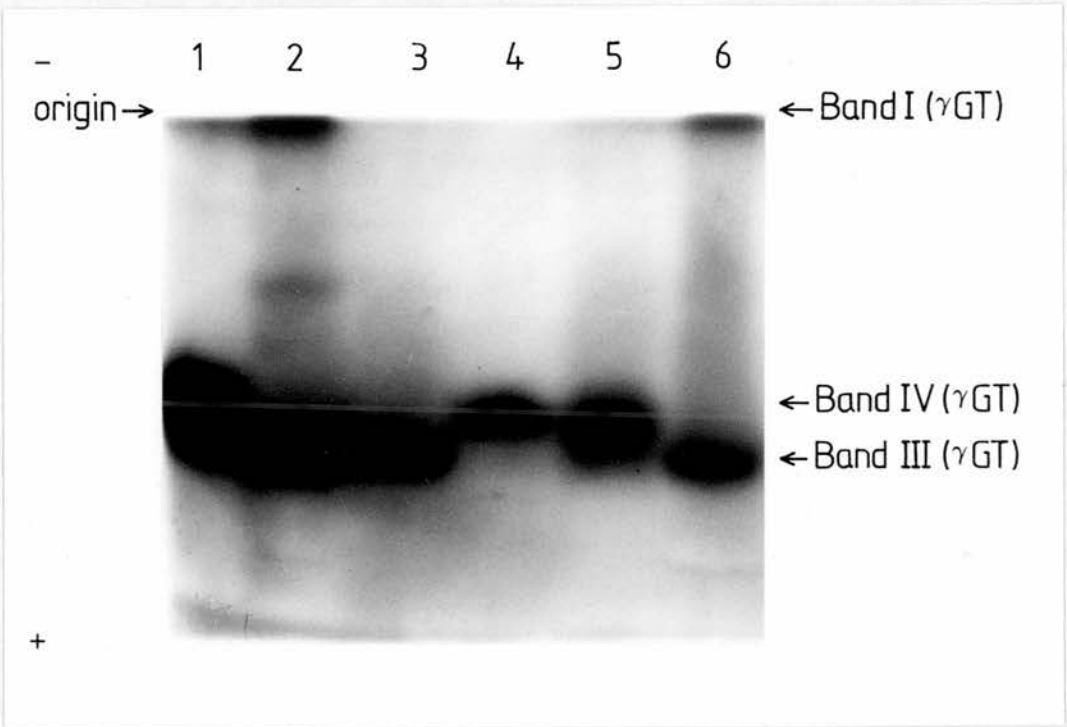


Figure 6.4 Polyacrylamide slab gel electrophoresis in 12 mmol/l sodium deoxycholate of γGT in deoxycholate and in papain-treated bile, liver microsomes and serum from a patient with extrahepatic biliary obstruction. 1, 4, 5, papain-treated bile, liver, serum; 2, 3, 6, deoxycholate-treated bile, liver serum.



6.1.6 Polyacrylamide gradient gel electrophoresis

The gradient gels were calibrated as described in section 2.4.4.

In the presence of 12 mmol/l deoxycholate, the electrophoretic mobilities, and therefore the estimated $\underline{M}_r$ values of the Peak 3 forms of γGT obtained from liver microsomes, bile and serum were identical (Fig. 6.5). The Peak 4 forms of γGT obtained from these three tissues also had identical mobilities (Band IV (γGT)), and travelled just ahead of the Peak 3 forms (Fig. 6.5). The estimated $\underline{M}_r$ values of the various fractions are shown in Table 6.1.

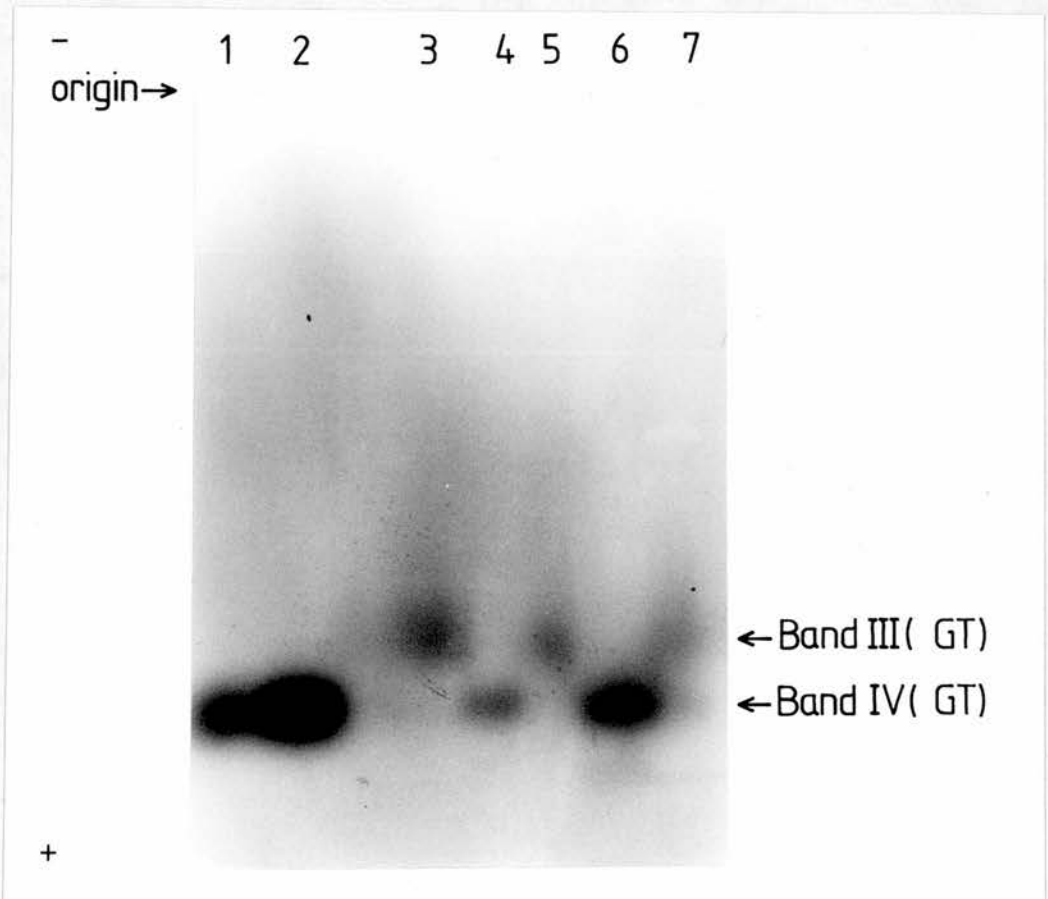
Table 6.1

Estimated $\underline{M}_r$ values of the enzyme fractions obtained following gel chromatography

Fraction	Estimated $\underline{M}_r$			
	Gel chromatography		Gradient electrophoresis	
	mean	range	mean	range
Peak 3	161 000	157 000 - 165 000	130 000	125 000 - 135 000
Peak 4	115 000	-	104 000	98 000 - 110 000

These values are in agreement with those of other workers for the Peak 3 and Peak 4 forms of γGT obtained from human liver (Huseby, 1977; 1978; Shaw et al., 1978; Echetebu and Moss, 1982a), bile and serum (Huseby, 1978). The slight differences between the $\underline{M}_r$ values for the Peak 3 and Peak 4 forms described here and those estimated by Sephacryl S300 gel chromatography (Table 4.4) are well within the experimental error of the methods.

Figure 6.5 Polyacrylamide gradient gel electrophoresis in 12 mmol/l deoxycholate of concentrated pools from Peaks 3 and 4 (γGT) obtained after gel chromatography. 1, Peak 4 (γGT) serum; 2, Peak 4 (γGT) bile; 3, Peak 3 (γGT) liver; 4, Peak 4 (γGT) liver; 5, Peak 3 (γGT) bile; 6, Peak 4 (γGT) serum; 7, Peak 3 (γGT) serum.



6.2 INCUBATION OF LIVER TISSUE IN VARIOUS FLUIDS

The aim of these experiments was to test the theory of elution (Moss, 1980) and attempt to reproduce the various γGT fractions observed in serum in liver disease, by an extension of the initial *in vitro* studies devised by Echetebu and Moss (1979).

Human liver tissue (50 g) was obtained at post mortem from a patient with no evidence of liver disease. The tissue was washed in ice-cold saline, cut into 1 g pieces and stored in plastic tubes at -60°C until required.

The incubation procedure was carried out largely according to Echetebu and Moss (1979). 4 ml of the appropriate fluid was incubated with 1 g of thawed liver tissue for 48 h at 37°C. At timed intervals, 0.2 ml aliquots were removed, centrifuged for 10 min at 2500 x *g*, assayed for γGT activity and stored at -60°C for electrophoretic studies. An equal volume of the fluid was then added to the incubation to maintain a constant volume. Correction was made for this addition when calculating the γGT activity. Sodium azide (0.5 g/l) was added to each fluid, prior to the incubation procedure, to inhibit bacterial growth. Sodium azide has been shown not to inhibit γGT activity (Echetebu and Moss, 1979) and this was confirmed in the present study.

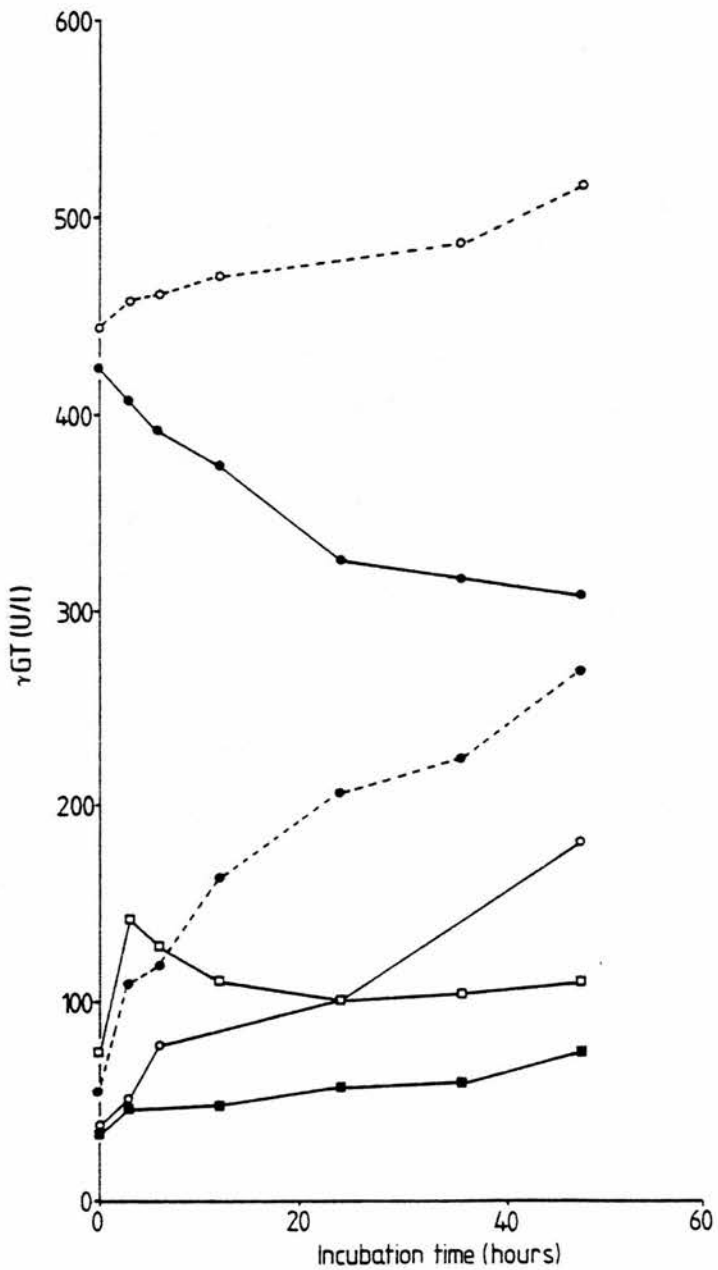
6.2.1 Incubation of liver tissue with a serum pool possessing 'normal' γGT activity

A normal serum pool, with a γGT activity of 45 U/l, was incubated for 48 h with liver tissue as described above. A control sample of the pool was also incubated for 48 h at 37°C in the absence of liver tissue.

During the incubation procedure, a steady increase was observed in the γGT activity of the liquid phase (Fig. 6.6).

At the end of the 48 h period, the liver-free supernatant and the control incubation were both subjected to gel chromatography on a 95 x 2.6 cm column of Sephacryl S300 calibrated as described in section 2.3.3. Using the

Figure 6.6 γGlutamyltransferase activity in the liquid phase after incubation of liver tissue with various fluids with increasing periods of time. The activity at time 0 was determined immediately after the addition of each fluid to the tissue. --●-- , normal serum pool; --○-- , abnormal serum pool; —○— , saline; —□— , 5 mmol/l glycyochenodeoxycholate; —●— , human bile; —■— , 60 g/l human albumin.



nomenclature described in section 4.3.1, the results (Fig. 6.7) show that the increase in γGT activity, observed in the supernatant from the incubation with liver tissue, is largely associated with an increase in Peak 1 (γGT), together with a smaller increase in Peak 4 (γGT), when compared with the control incubation. No apparent alteration in the amount of recovered activity of Peak 2 (γGT) was observed but its elution profile changed from that of Peak 2A (γGT) to that of Peak 2B (γGT), when compared to the control incubation.

Electrophoresis of the supernatants on polyacrylamide gradient gels confirmed the chromatographic findings. Incubation with liver tissue resulted, (1) in an increase in the band of activity at the origin (Band I (γGT)) which corresponds to Peak 1 (γGT) (section 4.4.1) and (2) in a decrease in the activity associated with Bands IIA (γGT), which correspond to Peak 2A (γGT), together with the appearance of Band IIB (γGT) which corresponds to Peak 2B (γGT) (section 4.4.1) (Fig. 6.8).

6.2.2 Incubation of liver tissue with serum pool possessing raised γGT activity

A serum pool, with a γGT activity of 402 U/l and containing approximately equal amounts of Peak 2A (γGT) and Peak 2B (γGT) (section 4.3.2), was obtained from patients with liver disease. This was incubated with liver tissue as described above and a control sample of the pool was incubated for 48 h without liver tissue.

Following incubation with liver tissue, a rise in the total γGT activity in the liquid phase was noted, but this was not as great as that observed in the normal serum pool (Fig. 6.6).

Gel chromatography on Sephacryl S300, of the liquid phase, obtained after 48 h incubation with liver tissue, showed an increase in activity associated with Peak 1 (γGT) compared with the control incubation (Fig. 6.9). This was accompanied by a decrease in the amount of activity eluting as Peak 2A (γGT).

Figure 6.7 Elution profile on Sephacryl S300 of γGT in the supernatant after incubation of normal serum pool with ● , or without ○ , liver tissue for 48 h at 37°C.

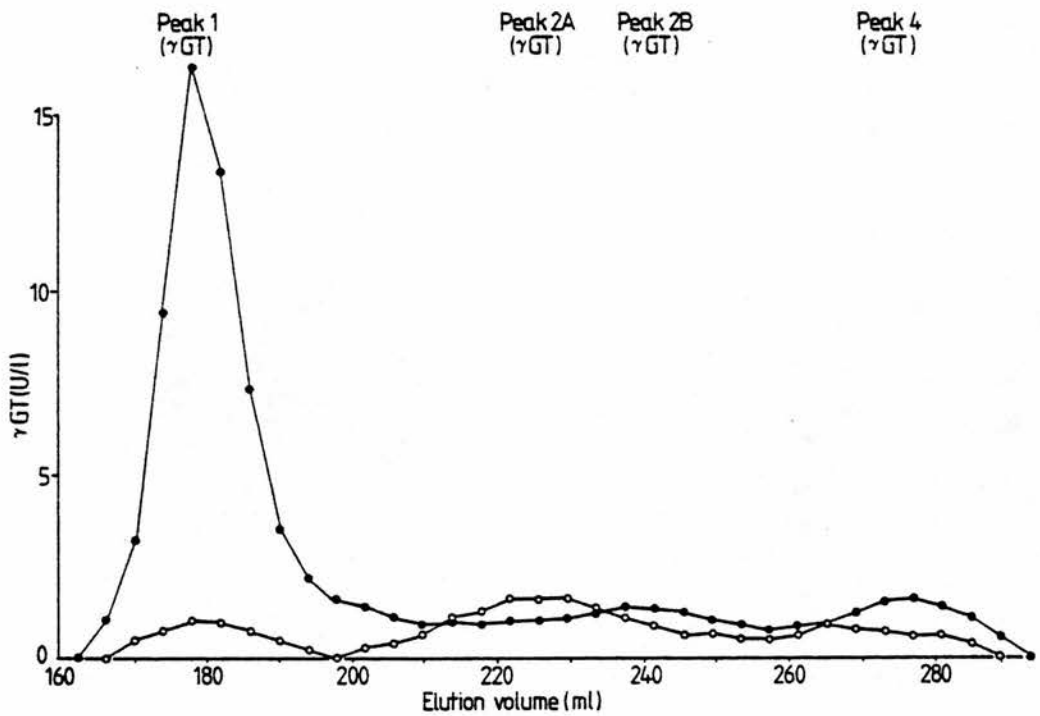


Figure 6.8 Polyacrylamide gradient gel electrophoresis of γGT in the supernatant obtained after incubation of a normal serum pool at 37°C for 48 h with or without liver tissue. 1, with; 2, without,

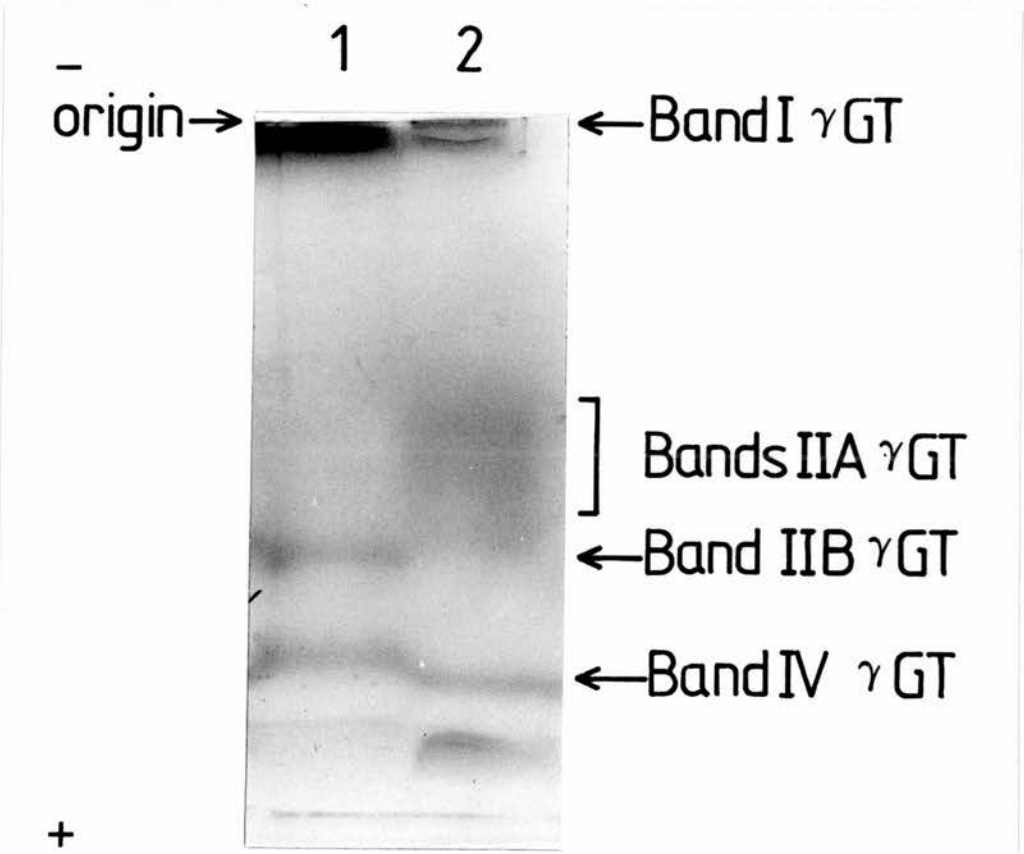
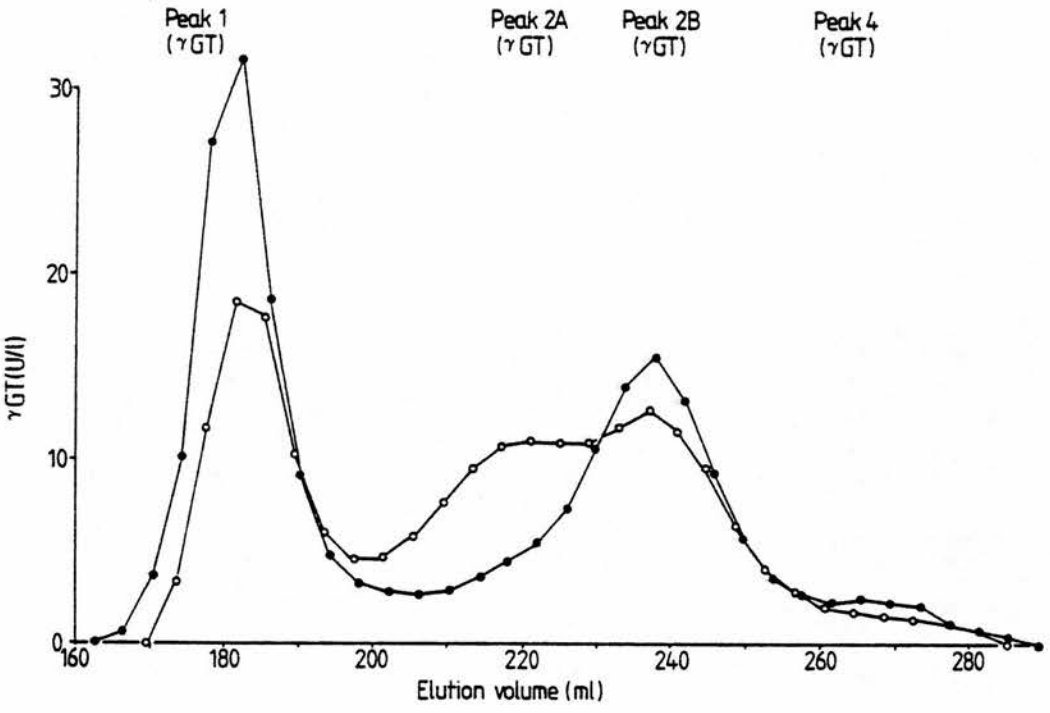


Figure 6.9 Elution profile on Sephacryl S300 of γGT in the liquid phase obtained after incubation of an abnormal serum pool with ● , or without ○ , liver tissue for 48 h at 37°C.



Electrophoresis of the 48 h supernatants on polyacrylamide gradients gave results that were in agreement with the chromatographic findings. Incubation with liver tissue resulted in an increase in the staining intensity associated with Band I (γGT), a decrease in the staining intensity associated with Bands IIA (γGT) and the appearance of a small zone of activity just ahead of Band IIB (γGT) (Band IID (γGT) (Fig. 6.10).

6.2.3 Incubation of liver tissue with saline

During incubation of liver tissue in saline for 48 h, a steady rise in the activity of the supernatant was observed (Fig. 6.6).

Gel chromatography of the supernatant on Sephacryl S300 demonstrated that the γGT released during the incubation procedure eluted as Peak 4 (γGT), (Fig. 6.11).

Polyacrylamide gradient gel electrophoresis of the supernatant, obtained at 0, 24 and 48 h after addition of saline to the liver tissue, showed a gradual increase in the intensity associated with Band IV (γGT) the electrophoretic mobility of which corresponds to that of Peak 4 (γGT) (section 4.4.1) (Fig. 6.12).

6.2.4 Incubation of liver tissue with 5 mmol/l glycochenodeoxycholate

Addition of 5 mmol/l glycochenodeoxycholate to liver tissue resulted in the immediate release into the liquid phase of more γGT activity than was evident in any of the other fluids in the incubation experiments. The activity reached a peak after 3 h and then fell to a slightly lower value for the remainder of the incubation (Fig. 6.6). This observation is consistent with leaching of the γGT from the plasma membrane into the liquid phase by the glycochenodeoxycholate, with solubilisation being complete after about 3 h. The fall in activity observed after this time may be due to slight instability of the enzyme, when maintained at $37^{o}C$ for long periods of time in an environment deficient of protein.

Figure 6.10 Polyacrylamide gradient gel electrophoresis of γGT in the supernatant obtained after incubation of an abnormal serum pool for different times at 37°C with or without liver tissue. 1, 36 h with liver; 2, 48 h with liver; 3, 48 h without liver.

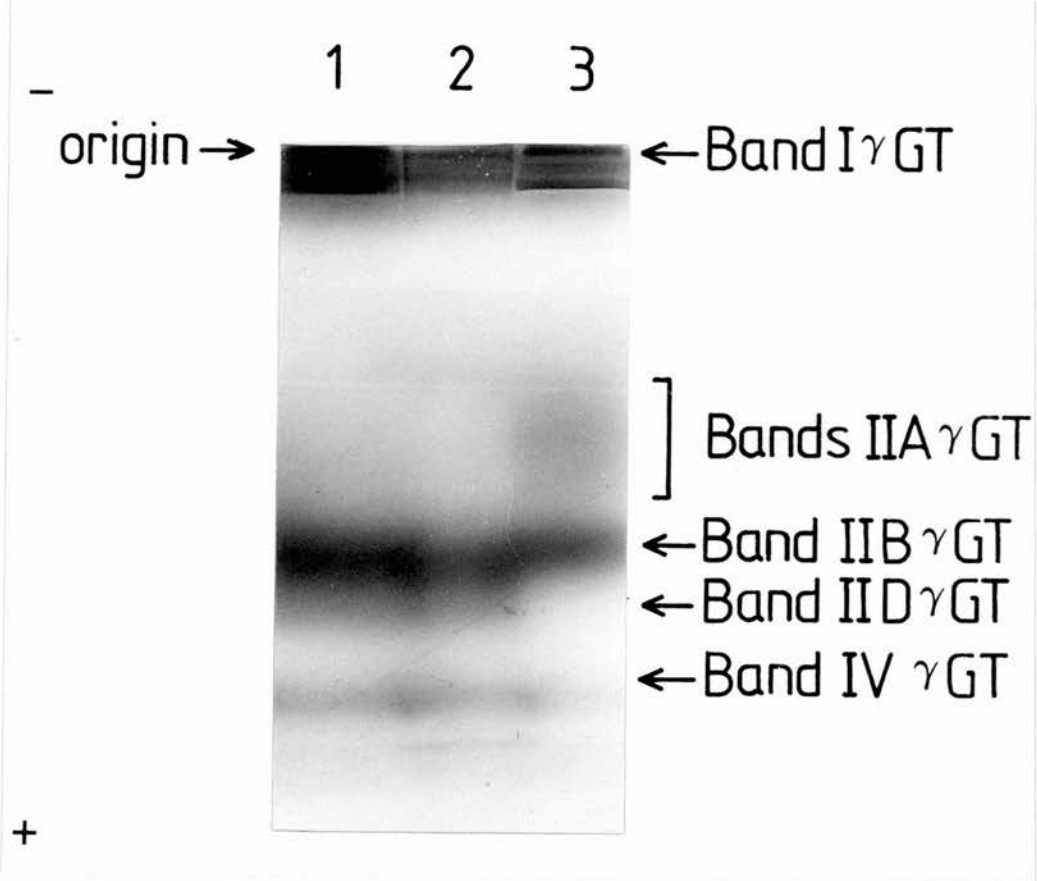


Figure 6.11 Elution profile on Sephacryl S300, of γGT in the liquid phase obtained after incubation of liver tissue in saline for 48 h.

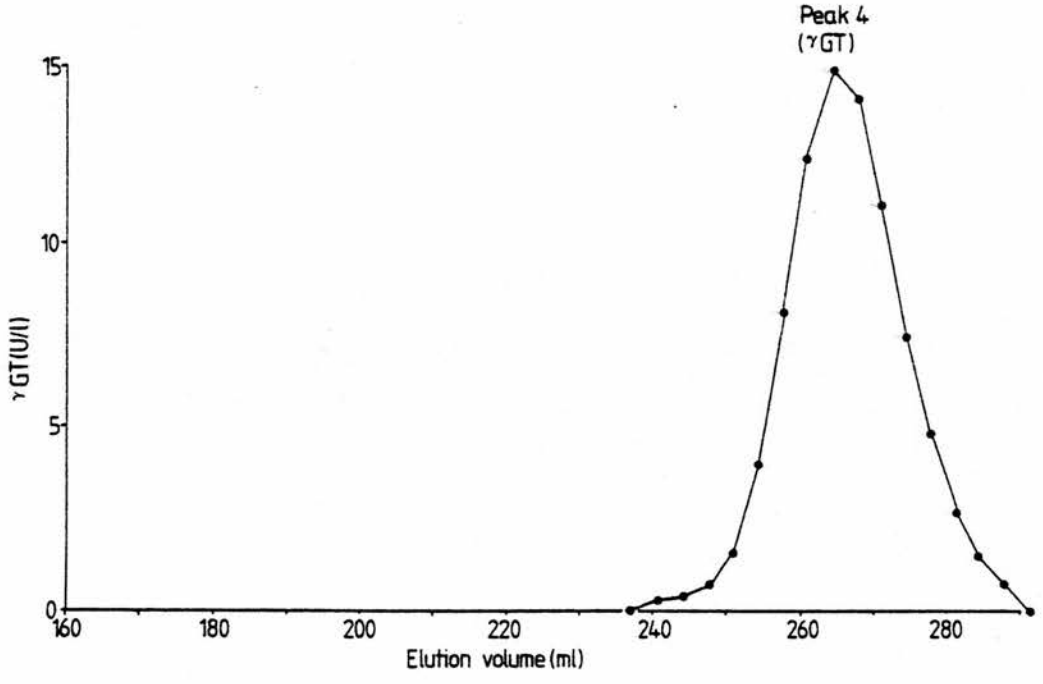
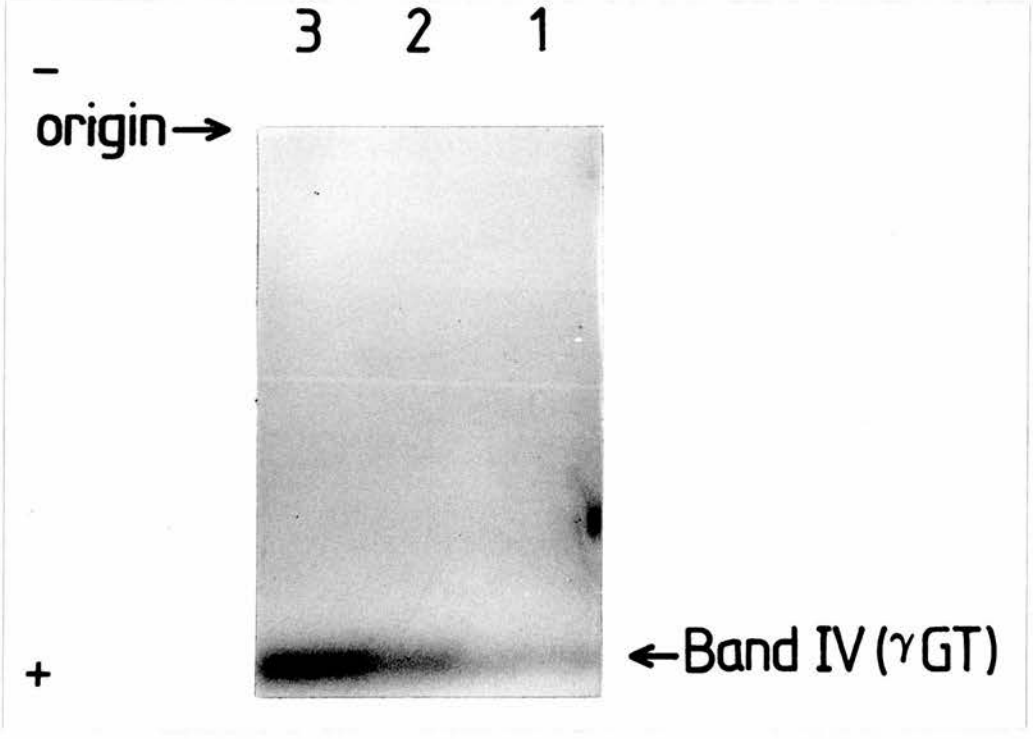


Figure 6.12 Polyacrylamide gradient gel electrophoresis of Peak 4 (γGT) and the supernatants obtained after incubation of liver tissue in saline for 24 and 48 h. 1, 24 h; 2, 48 h; 3, Peak 4 (γGT).



Gel chromatography of the 48 h supernatant in the absence of bile salts revealed a broad peak of γGT activity, suggesting partial reaggregation of low $\underline{M}_r$ hydrophobic enzyme during chromatography. When chromatography was repeated with 5 mmol/l deoxycholate in the elution buffer, a single peak of low $\underline{M}_r$ was obtained corresponding to Peak 3 (γGT) (Fig. 6.13).

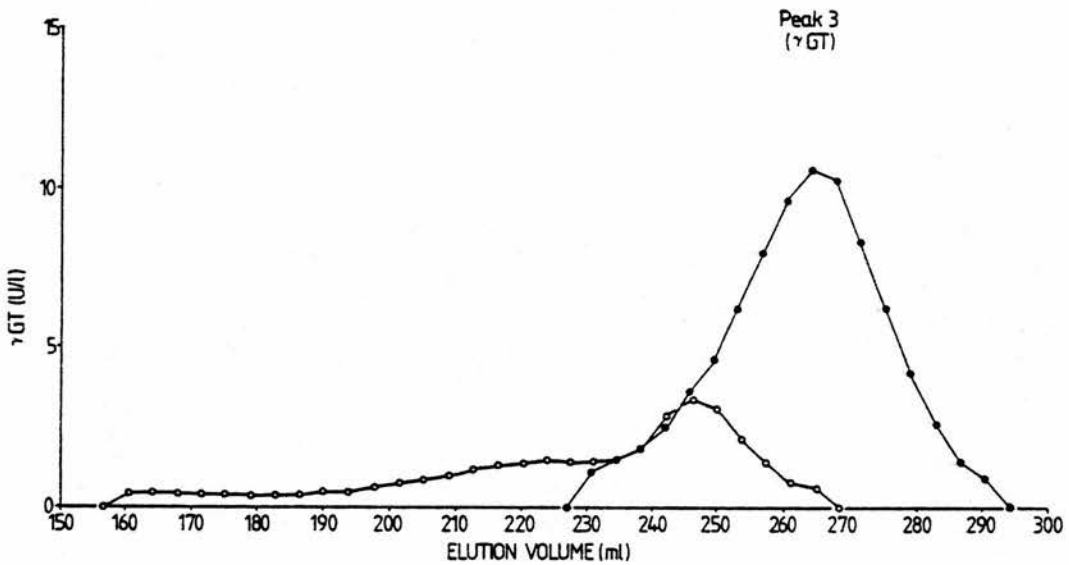
6.2.5 Incubation of liver tissue with human bile

Bile was obtained from a patient with 'T' tube drainage of the common bile duct. The concentration of total conjugated cholate was 6.5 mmol/l and of total conjugated chenodeoxycholate 1.5 mmol/l. The initial γGT activity was 439 U/l.

Following incubation of bile with liver tissue there was a steady decline in γGT activity in the liquid phase (Fig. 6.6).

Gel chromatography of the liquid phase obtained after incubation for 48 h with liver tissue revealed a decrease in activity of Peak 1 (γGT) compared to the control that had been incubated for 48 h in the absence of liver tissue (Fig. 6.14). This was accompanied by the appearance of a large peak of activity eluting immediately after Peak 1 (γGT). There was no apparent alteration in the small amount of Peak 4 (γGT) present in the native bile. Although the same volumes of 48 h supernatants were chromatographed, less activity was recovered from the liver incubate than the control; 0.69 U and 0.84 U respectively, with recoveries of applied activity of 87% and 95% respectively. This observation is in agreement with the fall in activity of the supernatant observed during the incubation procedure and suggests that γGT activity has been lost in the liver incubate, a finding which is difficult to explain. One possible explanation, advanced previously (section 6.2.4), is instability of γGT at 37°C in the absence of protein. Another, for which there is no proof, is that interaction of liver, or some component of liver, with biliary γGT somehow results in its inhibition. Gel chromatography of the liquid phase obtained after immediate mixing of the bile with the liver tissue revealed an almost identical elution profile to the supernatant obtained after 48 h incubation with the liver. The recovered activity of the former was greater, however, due to the higher γGT activity of the sample.

Figure 6.13 Gel chromatography of γGT in the supernatant, obtained after incubation of liver tissue with 5 mmol/l glycochenodeoxycholate for 48 h. ○ , no bile salts in the elution buffer; ● , 5 mmol/l deoxycholate in the elution buffer.



When the 48 hour supernatant obtained after incubation with liver tissue was chromatographed in the presence of 5 mmol/l deoxycholate, both Peak 1 (γGT) and the peak eluting immediately after it disappeared, giving rise to Peak 3 (γGT) (Fig. 6.14).

6.2.6 Incubation of liver tissue with human albumin

It was noted that incubation of liver tissue with normal or abnormal serum, i.e. solutions with high protein content, resulted in an increase in γGT activity which eluted as Peak 1 (γGT). In contrast, incubation of liver tissue with a protein-free solution, namely saline, resulted in release of activity which eluted as Peak 4 (γGT). In an attempt to see if this difference was due to the presence of protein in the incubation medium, a solution of 60 g/l albumin in 150 mmol/l NaCl was used in the incubation procedure.

The addition of the albumin solution to liver tissue caused the immediate appearance of, and a further steady rise in, γGT activity in the liquid phase (Fig. 6.6).

Gel chromatography of the 48 h supernatant on Sephacryl S300 revealed most of the γGT activity eluting as Peak 4 (γGT) with a small quantity eluting as Peak 1 (γGT) (Fig. 6.15).

Electrophoresis of the liquid phase on polyacrylamide gradient gels confirmed the chromatographic findings. There was a steadily, increasing intensity of Band IV (γGT) (Fig. 6.16).

6.3 DISCUSSION

The results of this preliminary study have confirmed the finding that γGT may be released from human liver microsomes by the bile salt sodium deoxycholate (Huseby, 1977, 1978; Shaw et al., 1978). In the presence of an adequate concentration of bile salts, the enzyme exists as a low M_r hydrophobic form (Peak 3 (γGT)) which reaggregates when the bile salts are removed, as

Figure 6.14 Gel chromatography on Sephacryl S300 of γGT in the liquid phase obtained after incubation of hepatic bile with □ , or without ○ , liver tissue for 48 h. ● , with liver but elution buffer equilibrated with 5 mmol/l sodium deoxycholate.

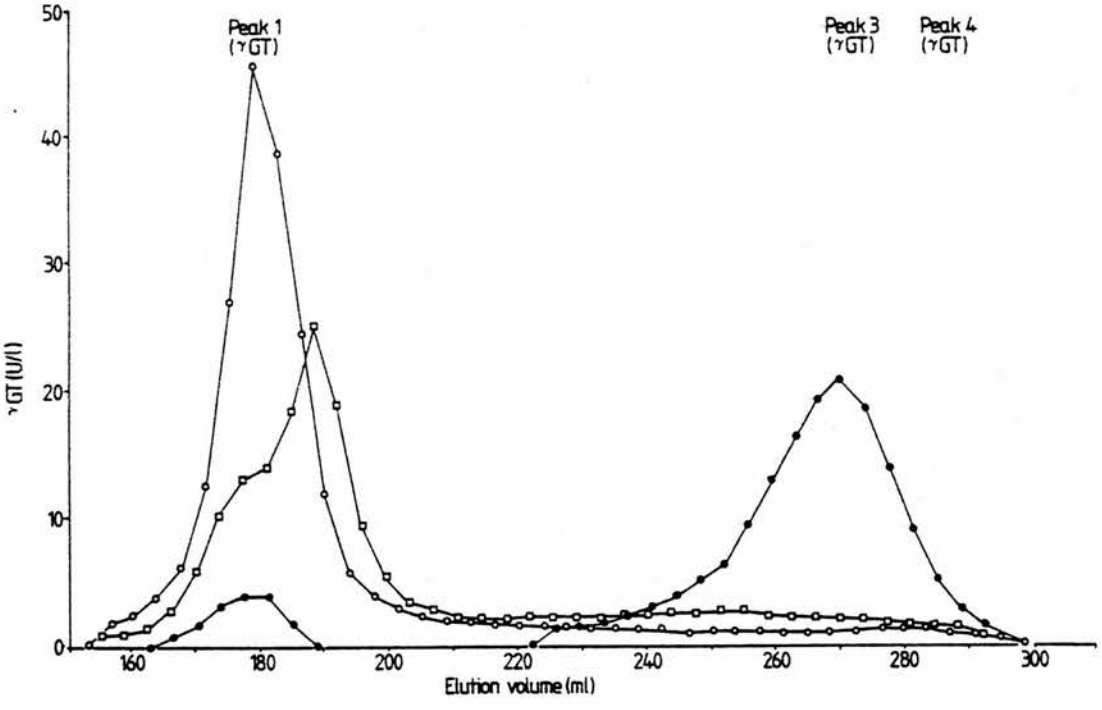


Figure 6.15 Elution profile on Sephacryl S300 of γGT in the liquid phase obtained after incubation of liver tissue in 60 g/l human albumin for 48 h.

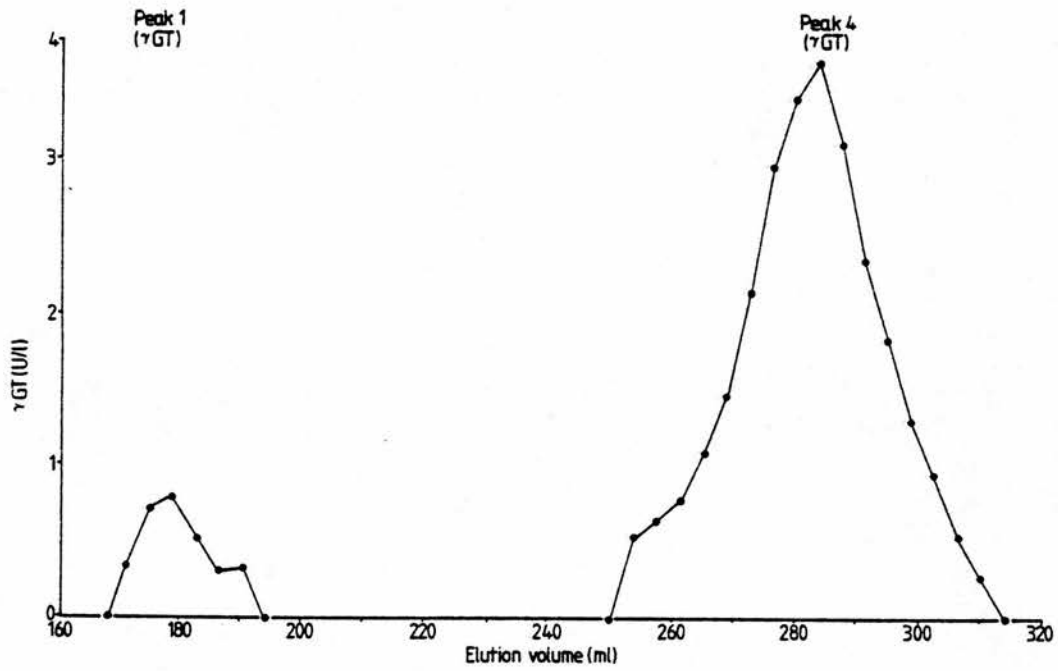
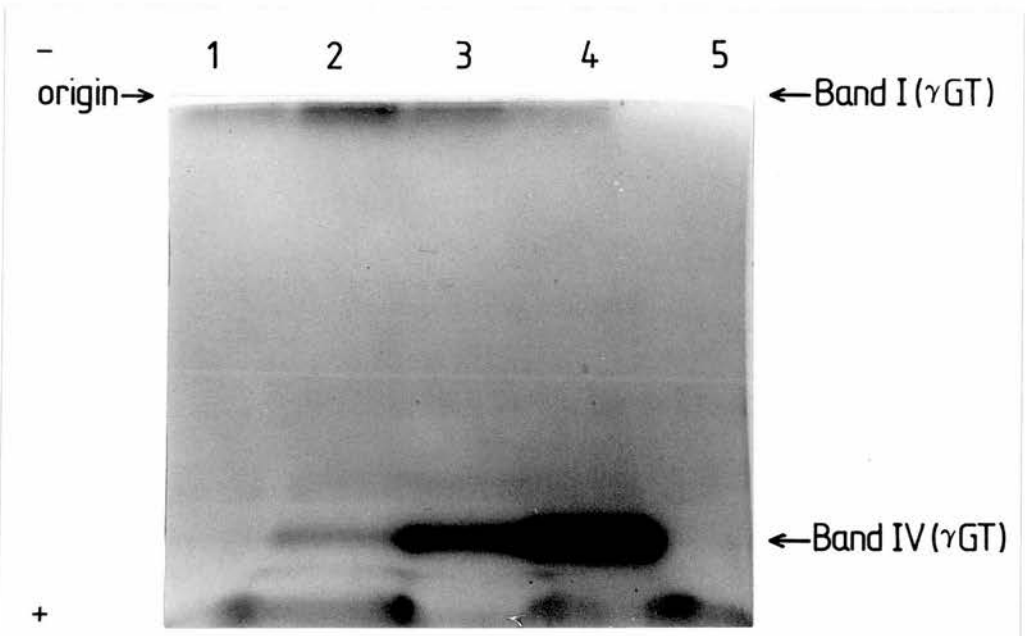


Figure 6.16 Polyacrylamide gradient gel electrophoresis of γGT in the liquid phase obtained after incubation of liver tissue in 60 g/l human albumin at 37°C for different periods of time. 1, 0 h; 2, 12 h; 3, 24 h; 4, 48 h; 5, Albumin solution 48 h, no liver.



observed by others (Huseby, 1978; Echetebu and Moss, 1982a). In contrast, the enzyme released by papain digestion (Peak 4 (γGT)), is hydrophilic in nature, shows no tendency to reaggregate and is of lower $\underline{M}_r$ than the Peak 3 form. It also appears to be produced by the action of trypsin on liver γGT (Shaw et al., 1978; Echetebu and Moss, 1982b).

The Peak 3 forms of γGT from human liver, bile (Chapter 3) and serum (Chapter 4) were all found to have identical electrophoretic and chromatographic properties. Similarly, the Peak 4 forms of γGT from all three tissues had identical physical properties. The implications of these findings are considered and discussed later in Chapter 7.

Liver tissue, when incubated with serum containing normal or elevated γGT activity, gave rise to γGT which was mainly in the Peak 1 (γGT) form and to much smaller amounts of the Peak 4 (γGT) form, confirming the results of Echetebu and Moss (1982a). In both these serum incubates there was a decrease in the activity eluting as Peak 2A (γGT) and in the normal serum incubate this was associated with an appearance of activity eluting as Peak 2B (γGT). The pathological serum incubate gave rise to a new fraction detectable only by electrophoresis (Band IID (γGT)) the significance of which is unknown at present. These findings, however, are at variance to those of Echetebu and Moss (1982a) who reported the complete disappearance of intermediate $\underline{M}_r$ γGT during the incubation procedure.

In contrast, incubation with a protein-free solution, such as saline, resulted in the release of Peak 4 (γGT), again confirming the work of Echetebu and Moss (1982a). However, incubation with protein-free or protein-deficient solutions containing high concentrations of bile salts, such as bile or a solution of glycochenodeoxycholate, resulted in the appearance in the liquid phase of γGT of hydrophobic nature, which appeared to undergo varying degrees of reaggregation during gel chromatography in the absence of bile salts.

The biochemical processes occurring during the incubation of liver tissue in the various fluids may, in part, be explained as follows: Incubation with protein-free solutions which are deficient in bile salts, such as saline or Ringer solution (Echetebu and Moss, 1982a), result in the generation within the liver

tissue of the hydrophilic Peak 4 form due to the action of intrinsic proteases. This hypothesis is supported by the observations of Huseby (1982b) who reported the conversion of hydrophobic γGT to a hydrophilic form during incubation of liver homogenates for 24 h at pH 7.0. This conversion was, 1) inhibited by the protease inhibitor diisopropylfluorophosphate, and 2) stimulated by the protease (and thiol) activator glutathione. In contrast, incubation with solutions deficient in protein, but containing high concentrations of bile salts (such as hepatic bile or glycochenodeoxycholate solution), result in the immediate release of the enzyme from the plasma membrane into the liquid phase. Once in the liquid phase, the enzyme is no longer in the environment of the proteases deep within the liver tissue and therefore remains hydrophobic in nature.

The suggested mechanisms for the in vitro γGT release from liver outlined above are in many ways incomplete. The in vivo mechanisms are even more obscure. Echetebu and Moss (1982b) suggested that incubation of liver tissue in serum results in the release of enzyme in complexed or particulate form, such as membrane fragments. If this suggestion is correct, then some factor in serum is able to stabilize these complexes and inhibit their hydrolysis by proteases. The results of this preliminary study have shown that the factor is not albumin and further studies are required to identify its exact nature and throw further light on the mechanisms involved.

Chapter 7

GENERAL DISCUSSION

The principle objective of this thesis was to investigate the physical and chemical nature of the multiple forms of γGT in human bile, serum and liver. The main interest was to determine from these studies, a) the biological origin and relationship to one another of these multiple forms and b) if their measurement in serum was likely to be of diagnostic value.

The results have been discussed in detail already and compared in the relevant chapters with similar experiments carried out by other authors. The aim in this chapter is to consider the findings on a wider basis and thus to build a unifying picture of the pathophysiological events occurring when γGT is released from the diseased liver cell and subsequently reaches the circulation.

7.1 ISOENZYMES OR MULTIPLE FORMS?

By definition, true isoenzymes, as opposed to multiple forms of an enzyme, are genetically different proteins. Multiple forms of an enzyme may arise, 1) by post-translational changes within the cell such as the addition of sialic acid residues to glycoproteins, 2) proteolytic modification after release from the cell, 3) association with other molecules, and 4) polymerisation of enzyme molecules. When the first description of γGT isoenzymes appeared, the distinction between isoenzymes and multiple forms was less rigid and, in fact, the authors may not have considered the point at all (Rutenburg et al., 1967; Jacyszyn and Laursen, 1968; Patel and O'Gorman, 1973). However, their findings must now be modified in the light of more recent work including that described in the present study.

7.1.1 Effect of detergents

Detergents have been used to solubilise membrane-bound proteins or protein aggregates with the minimum of damage. They are amphiphilic molecules composed of a hydrophilic head, which may be charged or neutral, and a hydrophobic tail, which may be either aliphatic or aromatic in nature. They are able to solubilise proteins because of their amphiphilic nature. Firstly, the non-polar tails interact with the hydrophobic region of the protein (which may itself be linked by hydrophobic interactions to the lipid-rich membrane). Secondly, the polar heads are able to form strong non-covalent bands with the surrounding water. These interactions result in an increase in entropy, making solubilisation favourable (Helenius and Simons, 1975).

The high and intermediate $\underline{M}_r$ hydrophobic forms of γGT in bile, serum and liver are each converted by bile salts to low $\underline{M}_r$ hydrophobic forms (Chapters 3, 4 and 6) (Peak 3 (γGT)). These low $\underline{M}_r$ forms, irrespective of their source, have similar chemical and physical properties and therefore there is strong circumstantial evidence that they are, in fact, identical. Treatment of bile (Chapter 3) or of serum (Huseby, 1978) with Triton-X 100 also converts the hydrophobic forms of γGT to lower $\underline{M}_r$ forms. All of these detergent-produced low $\underline{M}_r$ forms still possess hydrophobic properties and all reaggregate when in an environment deficient in detergent. Because of this property, a valid comparison of the γGT protein from the different tissues is not possible without the use of detergents.

7.1.2 Effect of proteolytic enzymes

This study has shown that papain treatment converts γGT from human serum, bile or liver to a low $\underline{M}_r$ hydrophilic form (Chapters 3, 4 and 6) (Peak 4 (γGT)). This low $\underline{M}_r$ form tends neither to aggregate nor to form complexes. Similar results obtained after treatment of γGT with proteolytic enzymes have been reported by others (Curthoys and Kuhlenschmidt, 1975; Huseby, 1977, 1978; Tate and Ross, 1977; Echetebu and Moss, 1982b). The most likely explanation of these findings is that hydrophobic forms of γGT possess a hydrophobic domain on the molecule. This domain causes γGT to aggregate or to become

attached to lipids. Removal of this domain by proteolytic enzymes renders the molecule unable to undergo such hydrophobic interactions.

7.1.3 Effect of neuraminidase

The enzyme neuraminidase (EC 3.2.1.18) removes sialic acid residues from glycoproteins. Electrophoresis of hydrophobic γGT from human serum, liver, kidney and pancreas in the presence of detergents (for the reasons stated above) showed that the mobility of the enzyme from liver is identical to that from serum but differs from that of the enzyme from both kidney and pancreas. After treatment with neuraminidase, however, the enzymes from all of these tissues have identical electrophoretic mobilities (Huseby, 1981). Electrophoresis of the hydrophilic forms of γGT obtained from human liver and from kidney showed that they too have different electrophoretic mobilities (Shaw et al., 1980). This finding is also consistent with each of these forms of the enzyme containing different numbers of sialic acid residues from one another.

7.1.4 Interaction of γGT with lectins

Lectins (Latin, legere, to select or pick out) are carbohydrate-binding proteins produced by plants. Broadly speaking, they may be divided into 4 groups (Goldstein and Hayes, 1978), based upon their carbohydrate specificity:

1) D-Mannose (D-glucose)-binding lectins, e.g. concanavalin A of the jack bean.

2) 2-Acetamido-2-deoxy-D-glucose-binding lectins, e.g. wheat germ lectin.

3) 2-Acetamido-2-deoxy-D-galactose-binding lectins, e.g. soybean lectin.

4) D-Galactose-binding lectins, e.g. castor bean lectin.

To a certain extent, differences between glycoproteins can be characterised by the lectin-binding properties of the glycoproteins. Studies with γGT from liver, pancreas and kidney have shown that the lectin-binding properties of the enzyme differ between tissues (Shaw and Petersen-Archer, 1979; Huseby, 1981). On the other hand serum γGT has similar lectin-binding properties to those of liver γGT. These findings suggest that the differences in γGT that exist between tissues are due in part, if not *in toto*, to differences in sialic acid content. Furthermore, they support the assumption that serum γGT is derived from liver.

7.1.5 Kinetic studies

If the multiple forms of γGT studied in this thesis are, in fact, true isoenzymes, i.e. forms which originate from separate structural genes, then it is quite likely that they will exhibit quantitative differences in their catalytic properties.

As a result of detailed initial-velocity kinetic studies, γGT preparations from liver, kidney, pancreas and duodenum were all shown to have different kinetic constants (Shaw *et al.*, 1980). These authors proposed that their findings were consistent with the idea that the various multiple forms each contained different numbers of carbohydrate residues. In contrast, in the present study, the kinetic constants of γGT in bile and in serum were shown not to differ significantly (Chapter 4). Furthermore, in a similar study, the kinetic constants of γGT in bile and in serum were shown to be the same as those of the enzyme obtained from human liver (Echetebu and Moss, 1982a). These findings, 1) support the concept that γGT from liver, bile and serum each contain equal numbers of carbohydrate residues, and 2) provide further circumstantial evidence that γGT in bile and in serum originates from the liver.

7.1.6 Immunoinhibition studies

The consensus of opinion in the literature is that the multiple forms of γGT from human liver, kidney, pancreas and serum all contain the same antigenic determinants. Szewczuk et al. (1977) prepared rabbit antiserum to human kidney γGT, using highly purified kidney γGT as the antigen. They found that the kidney antiserum inhibited kidney γGT activity by 77% and liver activity by 57%, indicating a high degree of cross-reactivity. In similar studies, using rabbit antisera towards human liver γGT, Shaw et al. (1980) noted that the activities of γGT from human liver, kidney, pancreas and serum were inhibited equally by the antiserum but that the activity of γGT from hog kidney was inhibited to a smaller extent. Following double immunodiffusion against antiserum raised against purified, papain-treated human liver γGT, Huseby (1981) observed a reaction of identity between the enzyme from liver, serum, kidney, urine and pancreas. However, in contrast to the above observations, the γGT-antibody complexes were all enzymatically active. This finding is consistent with the antibody being directed at an epitope far enough away from the active site to avoid steric hindrance of the reaction process.

In conclusion, the evidence considered above is consistent with the idea that the multiple forms of γGT in bile and in serum represent modifications, in one form or another, of liver γGT.

7.2 THE HEPATOCYTE PLASMA MEMBRANE

The entire surface of the hepatocyte is covered by the liver cell membrane, which is a complex organelle. When studied by transmission electron microscopy, it appears as a three-layered structure, consisting of two electron-dense lines between which is an area of low electron intensity. This was interpreted by Davson-Danielle (1952) as being representative of a phospholipid bilayer sandwiched between two protein layers. However, this model has recently been replaced by the more dynamic concept of the cell membrane, the fluid mosaic model (Singer and Nicholson, 1972). According to this idea, the cell membrane consists of a double asymmetrical layer of phospholipids with their polar heads orientated towards the outside and their

hydrophobic tails towards the inside of the membrane. A large number of membrane proteins and glycoproteins are arranged transversely across the lipid bilayer in a highly specific configuration. There they are believed to function as receptors, enzymes or trans-membrane carriers. There are two important elements in the membrane concept, namely, the fluid state of the phospholipid bilayer at body temperature and the lateral mobility of the inserted protein molecules. The liver cell membrane should therefore be considered to represent a continuously moving supramolecular complex rather than a static structure.

Anatomically, the hepatocyte plasma membrane consists of biliary and sinusoidal poles joined by a lateral region, which is contiguous with other hepatocytes. The sinusoidal and biliary poles are interspersed with numerous microvilli, whereas the lateral region is, on the whole, smooth although it does possess desmosomes and gap junctions. The relative surface areas for the sinusoidal, biliary and lateral surfaces have been estimated to be 72, 13 and 15% respectively (Blouin, 1977). The biliary pole, that forms part of the biliary canaliculus, is competely surrounded by tight junctions, separating it from the rest of the plasma membrane. The three anatomical areas of the membrane each appear to have distinct physiological functions. The sinusoidal surface is involved in the transport of metabolites to and from the circulation together with the interaction of hormones responsible for the regulation of hepatic function. The canalicular surface on the other hand is involved with the transport of bile into the canalicular space whilst the lateral region is largely concerned with intercellular communication (Evans, 1980).

γ-Glutamyltransferase and the other glycoprotein enzymes LAP, ALP and 5'NT are believed to comprise a group of 'ectoenzymes' that are located on the outer surface of the hepatocyte plasma membrane (Emmelot et al., 1968; Evans and Gurd, 1973; Evans et al., 1973; Wacker, 1974; Trams and Lauter, 1974; Gurd and Evans, 1974; Evans, 1974; Huseby, 1979). Although most workers agree that the enzymes are membrane-bound, there is conflict as to their location on the membrane surface. For example, in the rat, ALP has been reported, on the one hand as having only a sinusoidal (Hagerstrand and Norden, 1972) or, on the other hand, only a canalicular localisation (Ronchi and Desmet, 1973). Similarly, Naftalin et al. (1969), reported γGT in normal

human liver to be localised in the biliary canaliculi whereas Busachi et al. (1977) reported it to be present only on the sinusoidal surface.

7.3 THE NATURE OF γ-GLUTAMYLTRANSFERASE IN HUMAN BILE

Whereas many studies have been made of the content and output of bile salts, cholesterol, phospholipids and pigments in mammalian bile, biliary proteins and, in particular, biliary enzymes have aroused much less interest. Electrophoretic and immunological studies of human bile have confirmed the presence of many different proteins (Russell and Burnett, 1963; Wales et al., 1969; Englert, 1970; Dive and Heremans, 1974). A significant proportion of the biliary total protein is derived from serum proteins although biliary proteins occur at lower concentrations than, and in different relative proportions to, their concentration in serum (Mullock et al., 1978). On the other hand, some proteins, e.g. polymeric IgA-bound secretory component, are present at higher concentrations in bile than in serum. This bound polymeric IgA, together with free secretory component, makes a substantial contribution to the biliary protein profile (Lemaitre-Coelho et al., 1977; Mullock et al., 1978).

Many of the enzymes believed to originate from the hepatocyte membrane have been found in the bile of a large number of mammalian species (Holdsworth and Coleman, 1975; Coleman et al., 1979; Godfrey et al., 1981; Crofton and Smith, 1981a). In contrast, enzymes believed to originate from the cytosplasm of the hepatocyte are not detectable, or are present in low concentrations (Holdsworth and Coleman, 1975; Crofton and Smith, 1981a). It should be noted that most of the analytical techniques, such as gel chromatography or electrophoresis, used to study γGT and the other enzymes in bile, involve considerable dilution of the samples in aqueous solution. This, in effect, reduces the bile salt concentration in the solution containing the γGT. Under such analytical conditions, γGT and the other enzymes are present in solution as high M_r forms (Price and Sammons, 1974; Wenham et al., 1978; Huseby, 1978; Crofton and Smith, 1981a; Echetebu and Moss, 1982a). However, in the present study, it has been shown that if the analytical system is set up so that the γGT is maintained in an aqueous solution containing

physiological concentrations of bile salts, the enzyme is present as a low M_r form. Furthermore, the high and low M_r forms of γGT are interconvertible, depending on the bile salt concentration of the solution in which they are present.

In an attempt to explain the mechanism for the appearance of plasma membrane enzymes in bile, attention has long been centred on the possible involvement of bile salts. After infusion of taurocholate into rats, the biliary output of both 5'NT and ALP was increased (Javitt, 1965; Bode et al., 1973). In contrast, dehydrocholate, which does not form micelles, and secretin, which stimulates bile salt-independent bile flow, were less effective in increasing the biliary output of the enzymes (Bode et al., 1973). Experiments on the effect of various bile salts on human and sheep erythrocytes, and pig lymphocytes, have demonstrated the release of plasma membrane materials from intact cells before cell lysis (Holdsworth and Coleman, 1976; Billington et al., 1977; Billington and Coleman, 1978). Extending these studies to isolated rat hepatocytes, Billington et al. (1980) were able to release membrane-bound, but not intracellular enzymes, by incubation in different bile salts. High speed centrifugation of the incubation supernatant showed that, after incubation in 7 mmol/l glycocholate, 75 - 91% of the released enzymes was particulate, but when the glycocholate concentration was increased to 15 mmol/l, the proportion of the released enzymes in a particulate form fell to 45 - 75%. High speed centrifugation of rat bile results in 20 - 40% of plasma membrane marker enzymes being sedimented (Godfrey et al., 1981). Similar findings were obtained in the present study (Chapter 3) where it was also shown that the proportion of the plasma membrane enzymes sedimented fell with increasing bile salt concentration.

When all of these factors are considered, the balance of the evidence favours the following interpretation put forward by Simpson et al. (1984). At low bile salt concentrations, plasma membrane may be released initially in the form of small vesicles pinched off the tips of the microvilli of the canalicular membrane (Godfrey et al., 1981). However, as the concentration of bile salts in the biliary tract increases, the enzymes are leached from the particles and pass into solution.

7.4 HOW DOES SERUM γGT ACTIVITY RISE IN LIVER DISEASE?

The elevation of the serum activities of γGT and the other plasma membrane enzymes are largely associated with cholestatic disease of one form or another. Similar observations have been made in experimental animals, and, in particular, it has been found that ligation of the common bile duct of rats is a good model for cholestatic disease (Popper, 1968). Extensive studies have therefore been made using rats and other small animals in attempts to elucidate the mechanisms by which γGT and the other enzymes are released into the circulation. Furthermore, whereas in human patients with extrahepatic obstruction, interpretation may be made difficult because of secondary damage, in animals, such problems may be avoided by studying the changes that take place soon after obstruction. However, the results of animal experiments must be viewed with caution since the mechanisms in such experiments may differ from those in human disease.

Within 12 h of ligation of the bile duct of either rats or guinea pigs, the serum activities of γGT, LAP, ALP and 5'NT are all increased (Kaplan and Righetti, 1970; Righetti and Kaplan, 1972; Kryszewski et al., 1973; Mullock et al., 1977; Wooton et al., 1977; Huseby and Vik, 1978). The peak rises in activities do not all coincide with one another however. For example, in the study by Kryszewski et al. (1973), whereas the serum γGT activity showed a steady rise over 192 h, the rise in ALP activity peaked after 12 h and that of 5'NT after 24 h. It is difficult to decide, however, if these observations suggest different underlying mechanisms for each of the enzymes, since the same enzyme, in different studies, often took different times to reach the peak of its rise in serum activity.

Within 12 h of occlusion of the common bile duct, there is a marked rise in the specific activity of ALP in liver tissue (Kaplan and Righetti, 1970; Kryszewski et al., 1973; Simpson et al., 1984). This increase, and the accompanying rise in serum ALP activity described above, are largely, but not completely, prevented by actinomycin D, which inhibits transcription of DNA to RNA, and by cycloheximide, which inhibits the translation of messenger RNA into protein (Kaplan and Righetti, 1970; Righetti and Kaplan, 1972; Kryszewski et al., 1973; Huseby and Vik, 1978). In contrast, no significant changes in the

specific activities of γGT and 5'NT occurred until between 48 h and 192 h after ligation. Moreover, actinomycin D and cycloheximide did not affect the rapid rise in serum activity of these enzymes. The animals, however, could not be maintained long enough on these toxic compounds to study their effect upon the delayed rise in liver activity of these enzymes (Kryszewski et al., 1973).

The simplest interpretation of these findings is that increased synthesis of liver ALP is the cause of all or part of the immediate rise in serum ALP activity. However, Righetti and Kaplan (1972) also observed a small increase in hepatic activity 2 - 3 h after occlusion, followed by a later increase in serum activity, neither of which were prevented by cycloheximide. This suggests that part of the increased ALP activity in the circulation following bile duct ligation is not due to de novo protein synthesis.

In contrast to ALP, mechanisms other than protein synthesis appear to be responsible for the rapid rise in serum γGT and 5'NT activities seen in the period immediately following bile duct ligation in animals. One explanation for this would be simple regurgitation of bile through the damaged bile canaliculi or bile ducts (Popper, 1968). In the rat, after bile duct ligation, the bile canaliculi are first distended, then become tortuous due to the pressure of bile, resulting eventually in the opening up of canalicular-sinusoidal connections (Birns et al., 1962). Mullock et al. (1977) observed that 5'NT, in the bile and serum of bile duct-obstructed rats, bound to immobilised antiserum to rat liver plasma membrane. Having excluded the presence of plasma membrane fragments in the jaundiced sera, they concluded that the changes in serum 5'NT activity were consistent with the direct leakage of bile back into the circulation.

Membrane fragments, however, have been reported to be present in the circulation of patients with cholestasis (De Broe et al., 1975). This finding is also consistent with the occurrence of regurgitation, given that a significant proportion of the plasma membrane enzymes in human bile is believed to be present as a particulate form, with the remainder as a bile salt-solubilised, low M_r, hydrophobic Peak 3 form (section 7.3). The findings of the present study do not exclude the occurrence of regurgitation of biliary γGT. Regurgitation

of particulate γGT from bile could account for part of the rise in high $\underline{M}_r$ γGT activity observed in serum in cholestasis (Chapters 4 and 5). Similarly, regurgitation of the Peak 3 form into the circulation, where the bile salt concentration is an order of magnitude lower, would enable it to complex to any circulating hydrophobic molecule.

Another possible explanation for the appearance of the plasma membrane enzymes in the circulation in cholestasis is that it is due to solubilisation by bile salts. Righetti and Kaplan (1972) first suggested that the intrahepatic accumulation of bile salts observed in cholestasis might cause in vivo solubilisation of the enzymes. Huseby and Vik (1978) found support for this idea after noting that between 3 and 6 h after bile duct obstruction in the guinea pig, the serum γGT and total bile salt concentration correlated with one another. Furthermore, they calculated that the fall in specific activity of hepatic γGT could be accounted for by elution of the enzyme from the liver and passage into the circulation.

The results of the present investigation are consistent with the above hypothesis. In the present study, the percentage of high $\underline{M}_r$ γGT and of LAP and of ALP correlated with one another (Chapter 5). Furthermore, the highest levels of the high $\underline{M}_r$ forms were found in patients with extrahepatic obstruction, a group which also showed the highest serum levels of conjugated bile salts. One possible explanation of these findings is that a hydrophobic, but low $\underline{M}_r$ form of γGT (and LAP and ALP), is eluted from the hepatocyte plasma membrane by high intracellular concentrations of bile salts (Boyd et al., 1966; Greim et al., 1972). On passing into the circulation, where the concentrations of bile salts are lower than in the liver cell, the γGT aggregates either with itself or with other lipid containing particles (Huseby, 1982a). It has already been shown in Chapter 3 of this thesis, that high concentrations of bile salts are needed to maintain γGT in a hydrophobic low $\underline{M}_r$ form. The relatively poor correlation between high $\underline{M}_r$ γGT (%) and serum bile salt levels (Chapter 5) may be due to the fact that intracellular and serum bile salt concentrations do not always parallel one another. Further support for this idea comes from histochemical studies on human liver. In non-obstructive lesions (chronic aggressive hepatitis, alcoholic liver disease) large amounts of γGT activity were located in the bile ducts and canaliculi, but in severe cholestasis, few of

the bile ducts and periportal canaliculi stained for the enzyme (Busachi et al., 1977). Furthermore, these findings observed in cholestasis, could be reproduced by incubating sections of liver, containing large amounts of γGT activity, with increasing concentrations of bile salts.

This explanation is also consistent with the findings for high $\underline{M}_r$ LAP (%) and high $\underline{M}_r$ ALP % (Chapter 5). High $\underline{M}_r$ LAP (%) correlates well with high $\underline{M}_r$ γGT (%) and LAP is known to be readily accessible on the membrane surface, whereas high $\underline{M}_r$ ALP (%), which correlates poorly with high $\underline{M}_r$ γGT (%), is known to be less accessible to the dissociating action of, for example, papain, than LAP or γGT (Crofton and Smith, 1981b). It is therefore quite likely that ALP is much less accessible to the detergent action of bile salts than either γGT or LAP.

The rise in serum γGT activity seen in patients on long term anticonvulsant therapy or suffering from alcoholic liver disease, in contrast to those observed in cholestasis, has long been thought to be due to enzyme induction (Rosalki et al., 1971; Whitfield et al., 1973; Rosalki, 1976). Animal experiments have also played a part in attempts to elucidate the mechanisms behind the rise in serum γGT activity seen in these groups of patients. Administration of ethanol or phenobarbitone to rats produces a significant increase in the specific activity of γGT in hepatic tissues. These findings are consistent with the theory that the induction of hepatic γGT synthesis causes the rise in serum γGT activity observed in patients with alcoholic liver disease or on long term anticonvulsant therapy (Ideo et al., 1971; Straus et al., 1978). However, the induced enzyme is still largely present within the liver cell, attached to the plasma membrane and microsomal fractions, and so a rise in serum γGT activity will not be observed until the enzyme finds its way into the circulation (Ratanasavanh et al., 1979). More recent work, also involving experiments on animals, has suggested that this process may be facilitated by bile salts. After administration of phenobarbitone to rabbits, Ratanasavanh et al., (1981, 1982) reported a rise in both serum and liver γGT activity. However, no rise in serum activity was seen until after 18 days of phenobarbitone treatment, whereas a rise in liver activity was observed after 4 days. These authors also observed that phenobarbitone administration also facilitated bile salt-mediated release of γGT from isolated hepatocyte membranes

in vitro and caused an increase in the sphingomyelin content and, as a result, the phospholipid/cholesterol ratio of the membrane. Coleman et al. (1980) have reported that when membranes were incubated in glycocholate, those with the highest sphingomyelin/cholesterol ratio tended to lose more material in a soluble rather than a particulate form. When considering the alcoholic patient or the patient on long term anticonvulsant therapy, in the light of these findings, the occurrence of two processes can be envisaged. Firstly, there could be increased hepatic synthesis of the enzyme and secondly, an alternation in the chemical composition of the membranes, rendering their constituents more susceptible to bile salt-mediated release.

In conclusion, the above evidence is insufficient to confirm or exclude any of the current theories for the increase in serum γGT activity in liver disease. Although biliary regurgitation would be expected to contribute more towards the rise seen in cholestatic than in non-cholestatic lesions, it does seem likely that bile salts play an important role in determining the release of these plasma membrane enzymes into the circulation in liver disease. The exact processes, though, which facilitate the bile salt interaction with the hepatocyte plasma membrane and its components, remain to be determined.

7.5 γ-GLUTAMYLTRANSFERASE AS A LIVER FUNCTION TEST

The differential diagnosis of jaundice is a common diagnostic problem for the practising clinician whether in a District General or in a Teaching Hospital. Ideally, a test is required that is readily available, rapid, safe and accurate. At present however, there is no single technique that satifies all of these criteria.

The available techniques may be arbitrarily divided into two categories, invasive and non-invasive. Among the former are included endoscopic retrograde cholangiopancreatography, mini-laparotomy with cholangiography and very fine needle percutaneous transhepatic cholangiography. Included on the latter are scintography, sonography and computerised axial tomography as well as serum enzyme measurements.

The clinical study of the multiple forms of γGT described in Chapter 5 of this thesis had two limitations shared by almost all other similar studies. Firstly, it was not possible, in the limited period available, to follow the various diseases over their natural clinical course. Secondly, the number of patients suitable for study was limited by their availability and was therefore small. Nevertheless, the study showed that the electrophoretic measurement of serum Band IIB (γGT) was in most patients, able to distinguish extrahepatic from intrahepatic cholestasis. From the clinician's view, serum γGT electrophoresis, if provided as a service by the laboratory would be a safe, non-invasive method of investigation which most laboratories would have little difficulty in introducing.

In conclusion, serum γGT electrophoresis would be an extremely useful initial test to carry out in these patients and could sometimes render the more expensive and invasive tests unnecessary. However, it's exact place in the diagnostic sequence requires further study and investigation.

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PUBLICATIONS

The following enclosed papers have been published as a result of the work carried out for this degree:

Wenham P R, Horn D B, Smith A F. γ-Glutamyltransferases in bile and sera from patients with extrahepatic biliary obstruction. Clinica Chimica Acta 1981; 112: 113 - 122.

Wenham P R, Horn D B, Smith A F. The nature of γ-glutamyltransferase and other hepatocyte plasma membrane enzymes in human bile. Clinica Chimica Acta 1982; 124: 303 - 313.

Wenham P R, Horn D B, Smith A F. Physical properties of γ-glutamyltransferase in human serum. Clinica Chimica Acta 1984; 141: 205 - 218.

In addition, a further paper has been accepted for publication in Clinical Chemistry:

Wenham P R, Horn D B, Smith A F. Multiple forms of γ-glutamyltransferase: A clinical study.

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γ-Glutamyltransferases in bile and sera from patients with extrahepatic biliary obstruction

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Summary

γ-Glutamyltransferase has been studied in bile and sera from patients with extrahepatic biliary obstruction. In both fluids γ-glutamyltransferase activity is found largely in the high molecular mass fraction present in the void volume following G200 gel chromatography and which remains at the origin after 7% polyacrylamide gel electrophoresis.

When bile, sera and liver microsomes are treated with deoxycholate, a different form of γ-glutamyltransferase, with an approximate molecular mass of 157000 estimated by gel chromatography and of 125000 by gradient gel electrophoresis, is obtained. After treatment with papain, all three types of specimen gave rise to a form of γ-glutamyltransferase with a molecular mass of 115000 estimated by gel chromatography and of 98000 by gradient gel electrophoresis. The possible relationship between these various forms of γ-glutamyltransferase, and their relevance to the increases in activity in serum seen in extrahepatic biliary obstruction, are discussed.

Introduction

Although large increases in serum γ-glutamyltransferase (γGT; EC 2.3.2.2) activity are known to occur in extrahepatic biliary obstruction [1,2], the mechanism of the increases remains unknown. However, it has been generally assumed that in these patients the obstruction to bile flow causes the regurgitation into the plasma of the γGT normally present in bile.

It has been shown previously [3] that the increase in serum γGT activity in patients with biliary obstruction is due to the appearance of a high molecular mass, low density form of the enzyme. It has been suggested that this may represent either a fragment of liver cell membrane or a complex between γGT and a plasma lipoprotein, phenomena similar to those proposed for serum alkaline phosphatase in patients with biliary obstruction [4–6].

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In the present study further investigations have been carried out into some of the properties of the γGT present in bile and sera from patients with biliary obstruction. The γGT present in human liver microsomes has also been studied. The objective was to gain further insight into possible mechanisms for the increased serum γGT activity seen in patients with biliary obstruction.

Materials and methods

Sera with γGT activities varying between 192 U/l and 395 U/l were obtained from patients with extrahepatic biliary obstruction. Specimens of hepatic bile with γGT activity ranging from 250 U/l to 600 U/l were obtained from patients with percutaneous or "T" tube drainage of the common bile duct.

Samples of human liver were obtained post mortem from patients with no evidence or history of liver disease. The microsomal fraction of the liver homogenate was obtained from the 100000 × *g* pellet after cell debris and mitochondria had been removed by centrifugation at 3000 × *g* and 18000 × *g* respectively.

Chemicals

γ-Glutamyl *p*-nitroanilide was obtained from Boehringer, Lewes, Sussex; γ-glutamyl α-naphthylamide from Koch Light, Colnbrook, Bucks, U.K.; sodium deoxycholate, papain (twice crystallised) and Fast Blue B from Sigma Chemical Company, Poole, Dorset, U.K.; Sephadex G200 from Pharmacia, Uppsala, Sweden; acrylamide and bis-acrylamide from Eastman Kodak, Liverpool; carboxyl [^{14}C]sodium deoxycholate (52 μCi/mmol) from the Radiochemical Centre, Amersham, Bucks, U.K. All other chemicals were of analytical grade, purchased from BDH, Poole, Dorset, U.K.

Papain treatment

Samples of bile, serum and liver microsomes were incubated with papain (1 g/10 g protein) overnight at 20°C in the presence of 100 mmol/l cysteine.

Deoxycholate treatment

Bile and sera were incubated with an equal volume, and liver microsomes with a 10-fold volume, of 25 mmol/l sodium deoxycholate for 1 h at room temperature. The liver microsomes were centrifuged for 1 h at 100 000 × *g* following incubation, and the supernatant used.

7% Polyacrylamide gel electrophoresis

An RGA/500 electrophoresis apparatus (Raven Scientific, Haverhill, Suffolk, U.K.) was used with vertical gel slabs (140 × 165 × 3 mm) and a discontinuous buffer system of 180 mmol/l Tris/HCl, pH 8.9, gel buffer and 50 mmol/l Tris/glycine, pH 8.3, in the electrophoresis tanks.

Polyacrylamide gradient gel electrophoresis

The Uniscil electrophoresis apparatus (Universal Scientific Ltd., London, U.K.) was used with preformed polyacrylamide gel slabs with 4–30% gradient (Pharmacia, Uppsala, Sweden) in a continuous buffer of 50 mmol/l Tris/glycine, pH 8.3. Each gel was calibrated by running a mixture of standard proteins of known molecular mass [7].

Gel filtration chromatography

This was performed on Sephadex G200 using 20 mmol/l Tris/HCl buffer, pH 8.0, containing 50 mmol/l sodium chloride. A 650 × 26 mm column was used with an upward flow rate of 15.6 ml/h. Fractions of 2.6 ml were collected. The column was calibrated, for molecular mass determinations, using a mixture of proteins of known molecular mass. A linear semilogarithmic plot of molecular mass versus elution volume was obtained.

Localisation of γGT activity after electrophoresis

This was performed using γ-glutamyl α-naphthylamide as substrate [8].

Estimation of γGT activity

This was measured in serum, bile and column effluents at 37°C on an LKB 8600 reaction rate analyser [9]. The serum reference range for this method was 6–31 U/l for females and 8–49 U/l for males.

Results

Gel filtration chromatography

Depending upon the conditions of separation and on the type of sample applied to the column, 4 elution peaks of γGT were observed: Peak 1 eluted with the void volume, and therefore corresponds to a molecular mass greater than 600000; Peak 2 eluted at 115 ml, corresponding to a molecular mass of about 400000; Peak 3 eluted at 145 ml, corresponding to a molecular mass of about 157000; and Peak 4 eluted at 155 ml, corresponding to a molecular mass of about 115000.

Bile and sera from the patients with extra-hepatic biliary obstruction both showed an elution pattern in which Peak 1 predominated. Recovered activity was always greater than 85% and the percentage of total activity recovered in Peak 1 from both the bile and sera of patients with biliary obstruction ranged from 94% to 97% and from 56% to 77% respectively. Both showed small amounts of activity in Peak 4 (3–21%). In the case of sera, a significant amount of activity (18–37%) also eluted in Peak 2 (Fig. 1).

Following chromatography of deoxycholate-treated serum, bile and liver microsomes, with varying concentrations of deoxycholate in the eluting buffer, the main peak of γGT activity changed from Peak 1, when there was no deoxycholate in the eluting buffer, to Peak 3 when the deoxycholate concentration was more than 7 mmol/l. However, at intermediate deoxycholate concentrations, a significant amount of activity was present in Peak 2 with little or no activity in Peak 3 (Fig. 2).

After papain treatment, the specimens of serum, bile and liver microsomes all showed a similar pattern after gel chromatography, namely over 95% of the γGT activity eluted in Peak 4 (Fig. 3).

Papain treatment of the concentrated fractions from Peak 3 resulted in material with an elution pattern similar to that seen after papain treatment of the native samples, that is the activity was present in Peak 4. Treatment of any of the fractions from Peak 4 with 12 mmol/l deoxycholate did not result in any alteration in the elution pattern.

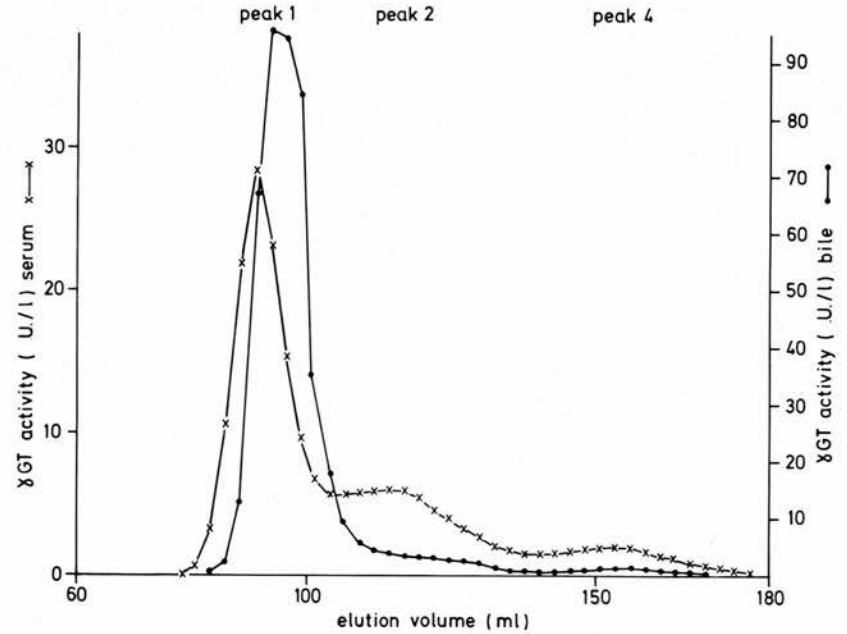

Fig. 1. Gel chromatography on Sephadex G200 of γGT present in serum from a patient with extra-hepatic biliary obstruction (× ——— ×), and in hepatic bile (● ——— ●).

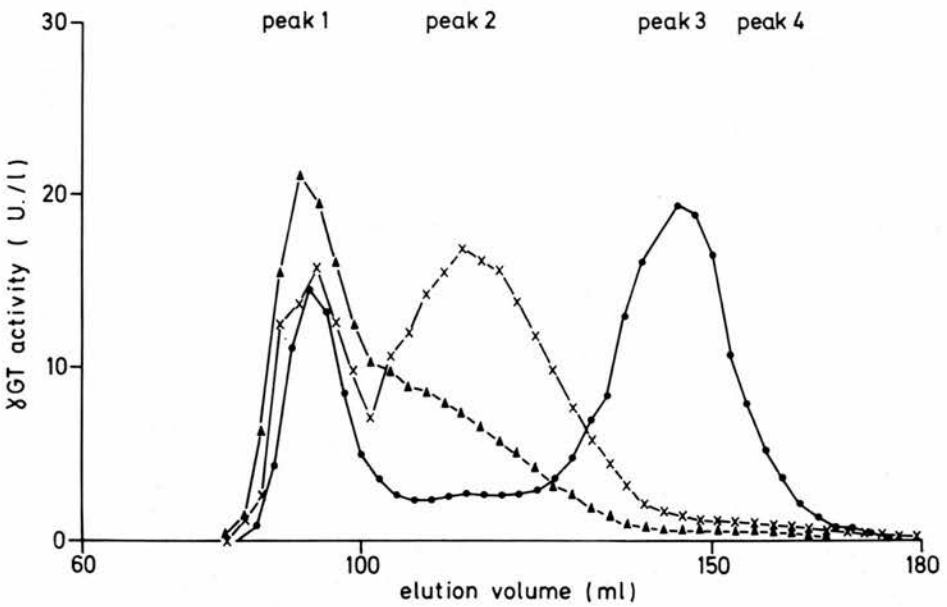

Fig. 2. Gel chromatography on Sephadex G200 of γGT in deoxycholate-treated hepatic bile; there were increasing concentrations of deoxycholate in the eluting buffer. No deoxycholate, ▲ ——— ▲; 1.2 mmol/l deoxycholate, × ——— ×; 7 mmol/l deoxycholate, ● ——— ●.

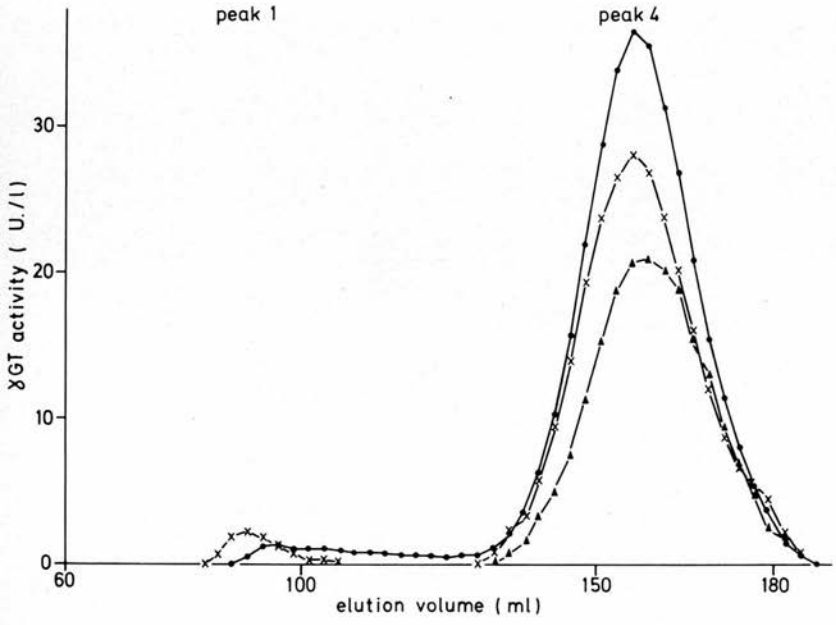

Fig. 3. Sephadex G200 gel chromatography of papain-treated γGT present in hepatic bile and serum from a patient with biliary obstruction and liver microsomes. Hepatic bile, ●——●; serum, ×——×; and liver microsomes, ▲——▲.

Polyacrylamide gel electrophoresis

The following results were obtained after electrophoresis in 7% polyacrylamide gel slabs:

(1) Untreated serum and bile both showed a major band of activity at the origin with a small amount of activity present in a band with 70% of the mobility of serum albumin. In serum, an additional band with mobility 50% of that of serum albumin (and identical mobility to that of a concentrate of Peak 2 obtained after gel chromatography) was also present (Fig. 4a), together with weakly staining zones of activity of slower mobility. Occasionally in bile, a weak additional band of mobility of 55% of serum albumin was observed.

(2) After treatment with deoxycholate, the results of electrophoresis depended on whether or not 12 mmol/l deoxycholate was also present in the gel buffer. When there was no deoxycholate in the gel buffer, the specimens of bile showed one or two additional bands with mobilities of 42% and 21% of that of albumin (Fig. 4b), and serum showed a long smear of activity. In the presence of deoxycholate, electrophoresis of specimens of liver microsomes, serum and bile, treated with deoxycholate, yielded a major "fast" band with mobility 84% of that of albumin although some activity remained at the origin (Fig. 5). The "fast" band had an identical mobility to that of a concentrate from Peak 3 obtained from gel chromatography.

(3) Electrophoresis of the papain-treated samples yielded a single band of γGT activity with mobility 70% of that of albumin (Fig. 5). This band had the same mobility as a concentrated pool from Peak 4, obtained after gel chromatography.

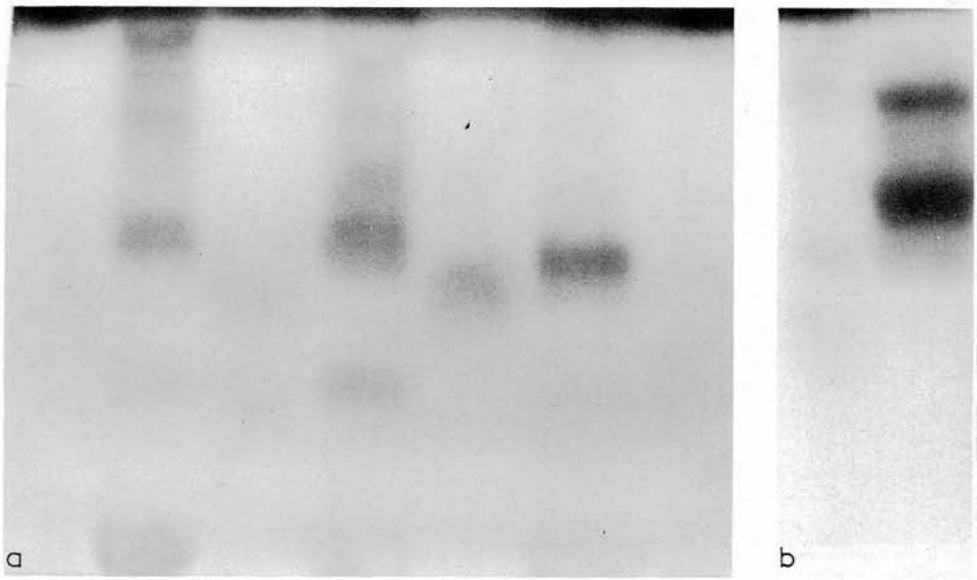

Fig. 4 (a and b). (a) Polyacrylamide slab gel electrophoresis of γGT in bile and serum from patients with extrahepatic biliary obstruction. Left to right, bile, serum, bile, serum, bile, serum, bile. The dark band at the lower (anodal) end of the serum samples is the albumin-bromophenol blue complex. (b) Polyacrylamide slab gel electrophoresis of γGT in bile before (left) and after (right) deoxycholate treatment.

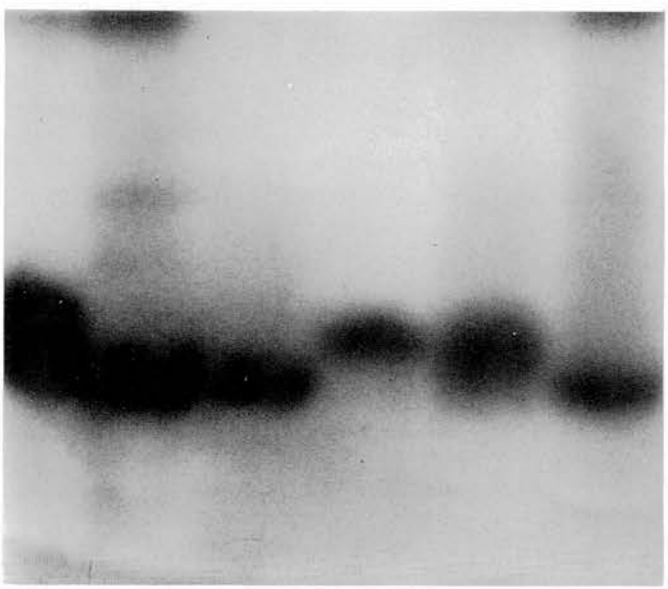

Fig. 5. Polyacrylamide slab gel electrophoresis in 12 mmol/l sodium deoxycholate of γGT in deoxycholate- and in papain-treated bile, liver microsomes and serum from a patient with extrahepatic biliary obstruction. Left to right, papain (bile), deoxycholate (bile), deoxycholate (liver), papain (liver), papain (serum), deoxycholate (serum).

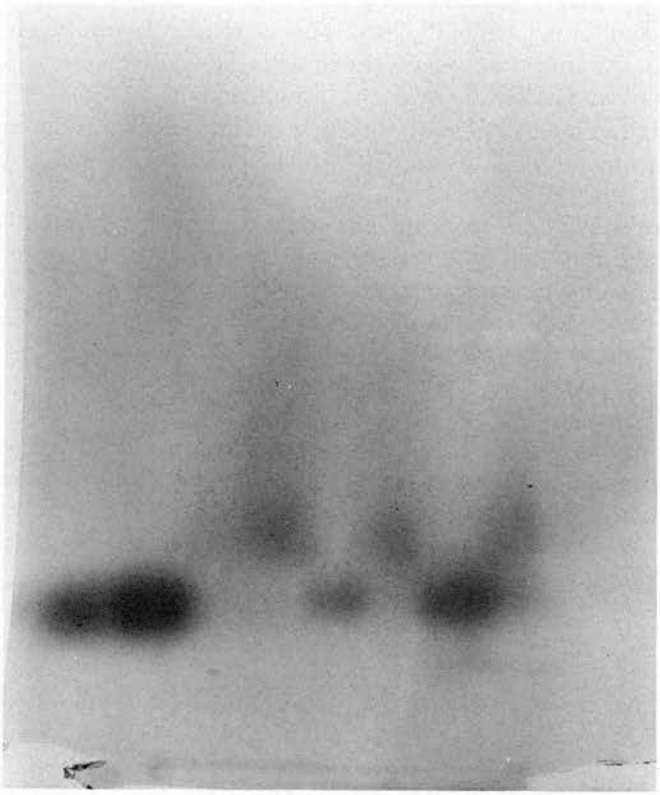

Fig. 6. Polyacrylamide gradient gel electrophoresis in 12 mmol/l deoxycholate of concentrated pools from Peaks 3 and 4 obtained after gel chromatography. Left to right, Peak 4 serum, Peak 4 bile, Peak 3 liver, Peak 4 liver, Peak 3 bile, Peak 4 serum, Peak 3 serum.

Polyacrylamide gel gradient electrophoresis

This yielded estimated molecular masses for Peaks 3 and 4 from gel chromatography of 125000 and 98000, respectively. It is noteworthy that, whereas Peak 4 had a greater mobility than Peak 3 in the gradient gel, these findings were reversed in 7% acrylamide gel. This suggests that the γGT in Peak 3 carries a much greater negative charge than the γGT in Peak 4 (Fig. 6).

[^{14}C]Sodium deoxycholate binding studies

These were restricted to bile, since bile has a higher γGT activity/g protein than serum. The experiments were designed to indicate whether bile salt binding to γGT could account for the differences in molecular size and charge between the γGT in Peaks 3 and 4.

(1) Neat bile (2 ml) was incubated for 1 h at room temperature with 5 μl of a solution containing 1 μCi [^{14}C]deoxycholate (19.2 nmol). This mixture was then chromatographed on Sephadex G200. Radioactivity (measured on a Packard Tri-Carb Liquid Scintillation Counter [10]) was present only in the salt volume peak and did not correspond with any of the peaks of the γGT activity.

(2) Bile was concentrated 20-fold on a Minicon macro solution concentrator (Amicon, London, U.K.). The concentrated bile was then incubated for 1 h with 1 μCi [^{14}C]deoxycholate. Column chromatography of the mixture on Sephadex

G200, which had been pre-equilibrated with [^{14}C]deoxycholate (5 μCi/l), resulted in three peaks of radioactivity. The first peak (A) co-eluted with the void volume, and the majority of the γGT activity. The second peak (B) of radioactivity eluted at 175 ml, corresponding to a molecular mass of about 70 000 and the third peak (C) eluted with the salt volume; there was no γGT associated with these peaks. The ratio of radioactivity in Peak B to that in Peak A was 1.45 : 1.

(3) The previous experiment was repeated using bile which had been treated with papain. A similar elution pattern to that observed following deoxycholate treatment was obtained, apart from a relatively greater amount of radioactivity appearing in Peak B (ratio of Peak B to Peak A was 1.83 : 1). The total amount of radioactivity in Peak B was approximately the same in both experiments.

Incubation of Peak 4 with bile and serum

Samples of bile and serum, each containing 0.24 U of γGT activity, were added to 0.5 ml of concentrated Peak 4 eluate, also containing 0.24 U of γGT. The volumes were made up to 2 ml with Tris/HCl buffer, pH 8.0, and the solutions incubated at 20°C for 6 h and 30 h. Following this, gel chromatography showed that the incubation had not altered the chromatographic behaviour of Peak 4.

Discussion

The present study confirms previous reports [3,11–13] that the γGT present in the sera of patients with biliary obstruction is mainly in a high molecular mass form, but with some enzyme of an intermediate molecular mass of about 400 000 (Peak 2) and a small amount with a molecular mass of about 115 000 also present. Biliary γGT showed similar properties on gel chromatography and polyacrylamide gel electrophoresis, apart from the absence of Peak 2, suggesting that it too is mainly of high molecular mass, in agreement with previous studies [14–15]. This conclusion must be viewed with caution, however, since the γGT in bile has been shown in this and in another study [16] to dissociate into a lower molecular mass form in the presence of bile salts or other detergents. The results presented here suggest that a bile salt concentration of about 7 mmol/l or greater is required to achieve this effect. Human bile has a much higher bile salt concentration than this, at least 10 mmol/l, although much of the bile salt would be present in micellar form. There is, therefore, a distinct possibility that the apparent high molecular mass of biliary γGT is an artefact caused by reaggregation of the enzyme when removed into a solution containing no bile salts.

Deoxycholate treatment of sera and bile from patients with extrahepatic biliary obstruction, and of the liver microsomal preparations, caused the formation of γGT with a molecular mass of 157 000 when estimated by gel chromatography (Peak 3) and of 125 000 when measured by gradient gel electrophoresis. This discrepancy in molecular mass may arise (i) from the glycoprotein moiety of γGT conferring anomalous behavioural properties on the enzyme towards gel filtration and electrophoresis [17,18], or (ii) due to difference in salt concentration between the two buffers causing differences in the configuration of the molecule. In the absence of bile salts, this molecule tended to reaggregate.

Papain treatment, on the other hand, caused the production of a γGT molecule which was smaller, with an estimated molecular mass of 115 000 by gel chromatogra

phy (Peak 4) and of 98000 by gradient gel electrophoresis. This γGT also carried a much smaller negative charge than the γGT resulting from deoxycholate treatment.

These findings are similar to those reported for γGT obtained from normal serum and bile [16] and for partially purified liver γGT following papain [19] or trypsin [20] treatment. Taken together, the findings in this paper are consistent with explanations which have been advanced previously, namely that bile salt or detergent treatment of γGT complexes results in the formation of a γGT molecule possessing a lipophilic area to which detergents may attach [16,21]. Papain treatment, on the other hand, may remove this lipophilic area, which is presumably a peptide or peptides, causing formation of a γGT molecule which will not bind to bile salts. It is perhaps not surprising that we were unable to confirm that radiolabelled deoxycholate binds to the lipophilic form of the enzyme, since the molar concentration of γGT in bile must be several orders of magnitude less than the concentration of radiolabelled deoxycholate required to obtain sufficient counts.

The mechanism for the rise in γGT activity in the serum of patients with extrahepatic biliary obstruction is uncertain. Firstly, low molecular mass (Peak 3) γGT might be regurgitated from bile into plasma, where subsequent reaggregation or binding to lipoproteins may occur. This is consistent with the previous finding that, although both bile and serum may contain a high molecular mass γGT, the two forms have different buoyant densities [3,15]. Secondly, biliary γGT may be in the form of membrane fragments which could be regurgitated into plasma in biliary obstruction; the differences in buoyant density [3,15] make this explanation less likely. Thirdly, the rise in bile salt concentration within the liver in cholestasis might cause "solubilization" of membrane-bound γGT, which might then gain direct access to the circulation and subsequently aggregate.

It seems much less likely that the elevated γGT activity found in serum during biliary obstruction could arise by proteolysis as has been suggested for normal serum [16,22]. Proteolysis would be expected to release the lipophobic form of γGT, which does not tend to aggregate, as shown by the experiments involving incubation of Peak 4 with bile and serum.

The fact that an intermediate molecular mass γGT has been detected (Peak 2) in sera from patients with obstructive jaundice could be taken as support for the thesis that reaggregation does, in fact, play a role in the mechanism. Such a form of γGT is not usually found in bile but does appear in the presence of relatively low concentrations of bile salts—conditions in which partial reaggregation could occur.

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The nature of γ-glutamyltransferase and other hepatocyte plasma membrane enzymes in human bile

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Summary

High molecular mass γ-glutamyltransferase in human bile is converted by physiological concentrations of the bile salts glycocholate and glycochenodeoxycholate to a form with a molecular mass estimated by gel chromatography to be 165000. A molecule of intermediate molecular mass estimated to be 330000 is obtained following treatment with the non-ionic detergent Triton X-100.

When human bile is centrifuged at $150000 \times g$, between 25–73% of γ-glutamyltransferase activity is recovered in the supernatant. This proportion is increased following prior addition of bile salts to the bile.

Analogous results are obtained for the high molecular mass enzymes alkaline phosphatase and leucine aminopeptidase, present in human bile, and also considered to originate from the hepatocyte plasma membrane. From these results it is suggested that these high molecular mass enzymes found in bile may, in part at least, represent artefacts following aggregation of the enzymes in aqueous media containing no bile salts.

Introduction

A number of enzymes such as γ-glutamyltransferase (γGT, EC 2.3.2.2), alkaline phosphatase (ALP, EC 3.1.3.1) and aminopeptidase (microsomal) (leucine aminopeptidase, LAP, EC 3.4.11.2), which are usually considered to originate from the hepatocyte membrane, are found in bile. However, the forms in which these enzymes are present in bile and the exact means whereby they pass into the bile are uncertain. Furthermore, it is not clear whether there is any relationship between the various

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forms of these enzymes as they are found in bile and the forms of the same enzymes found in increased amounts in the serum of patients with cholestasis.

Chromatography of bile on Sephadex G200 or similar materials demonstrates that all three of the enzymes mentioned above are apparently present mainly in a form with high relative molecular mass (M_r) [1–6]. However, it has been shown previously that, if sodium deoxycholate is included in the chromatography buffer, a low M_r form of γGT is recovered in the eluate [4,5]. This raises the question as to whether γGT (and also ALP and LAP) are present in bile in a low M_r form. It also suggests that the complexes found on gel chromatography may merely represent artefacts caused by aggregation of the enzymes in aqueous media in which no detergent, i.e. bile salt, is present.

In a previous study [5], we used the bile salt sodium deoxycholate in the chromatography buffer and studied only γGT. However, bile salts are normally present in bile as glycine or taurine conjugates and cholate and chenodeoxycholate conjugates are present in greater concentrations than those of deoxycholate. In order to mimic physiological conditions in bile more closely, we have now studied the effect of glycocholate and glycochenodeoxycholate on the elution pattern of γGT from Sephadex G200. We have included ALP and LAP in our study to determine whether our findings are more generally applicable, and have used electrophoresis, ultracentrifugation and treatment with the non-ionic detergent Triton X-100 to gain further insight into the nature of all of these enzymes in bile.

Materials and methods

Eight samples of hepatic bile were obtained from patients with 'T' tube drainage of the common bile duct. The range of enzyme activities were as follows; γGT, 100–1540 U/l, ALP, 75–1150 U/l and LAP, 22–126 U/l.

Chemicals

γ-Glutamyl-*p*-nitroanilide and *p*-nitrophenylphosphate were obtained from Boehringer, Lewes, Sussex, UK; γ-L-glutamyl-α-naphthylamide and L-leucyl-β-naphthylamide from Koch Light, Colnbrook, Bucks, UK; sodium glycocholate, sodium glycochenodeoxycholate, papain (twice crystallised), Fast Blue B, L-leucyl-*p*-nitroanilide, α-naphthyl acid phosphate and 4-aminodiphenylamine diazonium sulphate from Sigma Chemical Company, Poole, Dorset, UK; Sephadex G200 from Pharmacia, Uppsala, Sweden; acrylamide and bis-acrylamide from Eastman Kodak, Liverpool, UK. All other chemicals were of analytical grade, purchased from BDH, Poole, Dorset, UK.

Papain treatment

This was performed as previously described [5].

Treatment with bile salts

Prior to gel chromatography or electrophoresis, bile salts, equal to the concentration employed in the appropriate buffer, were added to bile. The bile was then stored at 4°C for 12 h prior to further investigation.

7% Polyacrylamide gel electrophoresis

This was performed in vertical gel slabs (140 × 165 × 3 mm) in the RGA/500 electrophoresis apparatus (Raven Scientific, Haverhill, Suffolk, UK). Electrophoresis of γ-GT and LAP was carried out using a modification [5] of the method of Azzopardi and Jayle [7]. Electrophoresis of ALP involved a modification [8] of the method of Kaplan and Rodgers [9].

Polyacrylamide gradient gel electrophoresis

4–30% polyacrylamide gradients (Pharmacia, Uppsala, Sweden) were used with the Uniscil electrophoresis apparatus (Universal Scientific Ltd., London, UK). A continuous buffer system was always used, being 50 mmol/l Tris/glycine, pH 8.3, for γGT and LAP, and 370 mmol/l Tris/borate, pH 9.5, for ALP.

Localisation of enzyme activities following electrophoresis

γGT was localised using γ-glutamyl-α-naphthylamide as substrate [10], ALP with α-naphthyl acid phosphate [6] and LAP with L-leucyl-β-naphthylamide [6].

Gel chromatography

This was performed on a 650 × 26 mm column of Sephadex G200, calibrated with proteins of known molecular mass, as previously described [5].

Ultracentrifugation

Samples of bile (before and after treatment with bile salts) (10 ml) were centrifuged at 2500 × *g* for 10 min. 8.5 ml of the supernatants were then centrifuged for a further 1 h at 150000 × *g*. The supernatant was collected, the pellet washed with 1 ml 20 mmol/l Tris/HCl buffer, pH 8.0, and then resuspended in 2 ml of the same buffer. Enzyme activities were determined on all fractions.

Enzyme activities

These were estimated on a LKB 8600 Reaction Rate Analyser. The substrate for measuring γGT activity was γ-glutamyl-*p*-nitroanilide [11], for measuring ALP activity, *p*-nitrophenylphosphate [12] and for measuring LAP activity, L-leucine-*p*-nitroanilide [13]. The coefficients of variation were 5.2% within batch and 5.4% between batch for γGT at a mean value of 135 U/l, 2.4% within batch and 3.0% between batch for ALP at a mean value of 112 U/l and 3.9% within batch and 4.9% between batch for LAP at a mean value of 185 U/l.

Enzyme activities of column fractions were estimated once on 0.5 ml of each sample, the final reaction conditions being the same as those for enzyme activity measurement in bile. Coefficients of variation were 2.5% within batch and 4.0% between batch for γGT at a mean value of 7.6 U/l, 6.0% within batch and 6.5% between batch for ALP at a mean value of 1.3 U/l and 5.7% within batch and 7.9% between batch for LAP at a mean value of 3.8 U/l.

The enzyme fractions, represented by the peaks obtained by gel chromatography, were measured by determining the peak areas and the problem of overlapping peaks was overcome by extrapolation of the main peaks.

Measurement of bile salt concentrations

The concentration of conjugated cholate and conjugated chenodeoxycholate in bile was measured by radioimmunoassay [14,15]. Coefficients of variation were 7.0% within batch and 8.0% between batch for both methods at a mean concentration of 3.0 μmol bile salt/l.

Results

Effect of bile salts upon enzyme activities

Sodium glycocholate at concentrations up to 20 mmol/l had no effect upon γGT activity, whereas ALP activity appeared to be stimulated slightly. Sodium glycochenodeoxycholate, on the other hand, did not alter the activity of either of these enzymes at concentrations up to 10 mmol/l. Both bile salts significantly inhibited LAP activity (Table I).

Effect of papain treatment

Digestion of hepatic bile with papain resulted in a slight decrease in γGT activity, while the effect upon LAP activity was variable (Table II). In sharp contrast, ALP activity was destroyed following papain treatment.

Gel filtration chromatography

A fairly clear pattern of results has emerged from these investigations. In order to simplify the description, and agree with previous arbitrary nomenclature [5], we have named the peaks separately in the order of their elution from the gel column:

TABLE I

ENZYME ACTIVITIES FOLLOWING ADDITION OF BILE SALTS TO HUMAN BILE

	Percentage initial enzyme activity		
	γGT	ALP	LAP
Added glycocholate (mmol/l)			
0 *	100	100	100
5	100	117	86
10	102	131	73
15	102	131	59
20	104	139	49
Added glycochenodeoxycholate (mmol/l)			
0 *	100	100	100
1	95	92	83
3	94	97	68
5	94	108	59
10	93	110	42

* The endogenous concentration of conjugated cholate was 2.7 mmol/l and of conjugated chenodeoxycholate 0.5 mmol/l.

TABLE II

ENZYME ACTIVITIES AFTER DIGESTION OF HUMAN BILE WITH PAPAIN

	Percentage initial activity		
	γGT	ALP	LAP
Bile A	93	3	108
Bile B	78	0	74
Bile C	94	12	84

Peak 1: void volume for all enzymes.

Peak 2T (γGT), Peak 2T (ALP), Peak 2T (LAP): major intermediate M_r peaks eluting when 0.1% (w/v) Triton X-100 is present in the elution buffer. See Table III for estimated M_r values.

Peak 2T (iγGT), Peak 2T (ALP), Peak 2T (LAP): major intermediate M_r peaks eluting when 0.1% (w/v) Triton X-100 is present in the elution buffer. See Table III for estimated M_r values.

Peak 3 (γGT), Peak 3 (ALP), Peak 3 (LAP): major low M_r peaks eluting when bile salts are present in the eluting buffer. These peaks reaggregate in the absence of bile salts.

Peak 4 (γGT), Peak 4 (ALP), Peak 4 (LAP): minor peaks eluting after the corresponding Peak 3 and found in small amounts in native bile, and in large amounts (for γGT and LAP) in bile which has been previously treated with papain. These peaks do not undergo reaggregation in the absence of bile salts.

TABLE III

ESTIMATED MOLECULAR MASSES OF THE ENZYME FRACTIONS OBTAINED FOLLOWING GEL CHROMATOGRAPHY

Fraction	Estimated molecular mass					
	gel chromatography			gradient electrophoresis		
	γGT	ALP	LAP	γGT	ALP	LAP
Peak 2T	333000	400000	430000	–	–	–
Peak 3	165000	290000	310000	135000	280000	–
Peak 4	115000	200000	200000	110000	180000	177000

Chromatography performed in the absence of bile salts

After gel chromatography of native bile, between 87 and 93% of γGT activity eluted in the void volume, the remainder eluting as peak 4 (γGT), confirming our previous findings [5]. Similarly, both ALP and LAP eluted mainly as void volume peaks (Fig. 1) comprising 83–98% and 61–76% respectively of total recovered activity, the remainder eluting as Peak 4 (ALP) and Peak 4 (LAP). Recovery of γGT and LAP ranged from 80–100% and for ALP from 100–220%. The high recovery for ALP may possibly be due to the presence of low M_r inhibitors of ALP present in native bile, but separated from the enzyme as it passes through the gel column.

Chromatography performed in the presence of bile salts

Gel chromatography with increasing concentrations of glycocholate in the elution buffer resulted in a decrease in the amount of γGT eluting in the void volume, and the appearance of a fraction eluting as Peak 3 (γGT). Between 41% and 56% of γGT activity eluted as Peak 3 (γGT) (Fig. 2) when the elution buffer contained 20 mmol/l glycocholate. At this concentration of glycocholate the proportion of ALP activity eluting as Peak 3 (ALP) ranged from 32–45%. Results for LAP could not be obtained because at high concentrations of glycocholate the enzyme was almost completely inhibited during its passage through the column.

When glycochenodeoxycholate was included in the elution buffer, at increasing concentrations, the main peak of activity changed from Peak 1 to Peak 3 for all three enzymes. When the concentration was 5 mmol/l, 81–95% of γGT, 73–77% of ALP and 63–87% of LAP activity eluted as Peak 3 (Fig. 3). At intermediate concentrations of glycochenodeoxycholate significant amounts of activity of all three enzymes eluted as Peak 2, with little or no activity in Peak 3.

Chromatography performed in the presence of Triton X-100

2 vols. of bile were mixed with 1 vol. 0.3% (w/v) Triton X-100 and stored at 4°C overnight. Gel chromatography of the treated bile with 0.1% (w/v) Triton X-100 in

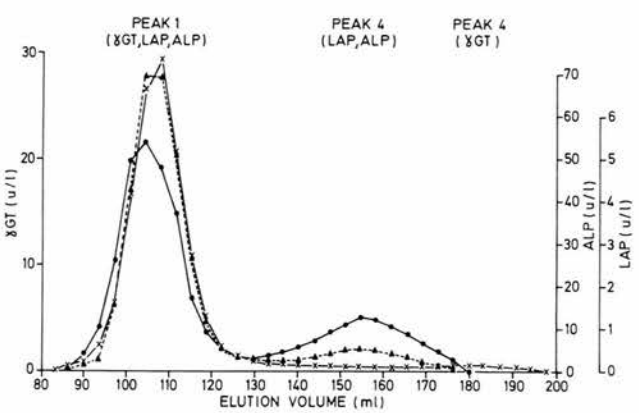

Fig. 1. Elution profiles of high M_r enzymes in human bile following gel chromatography on Sephadex G200. ×———×, γGT; ▲------▲, ALP; ●———●, LAP.

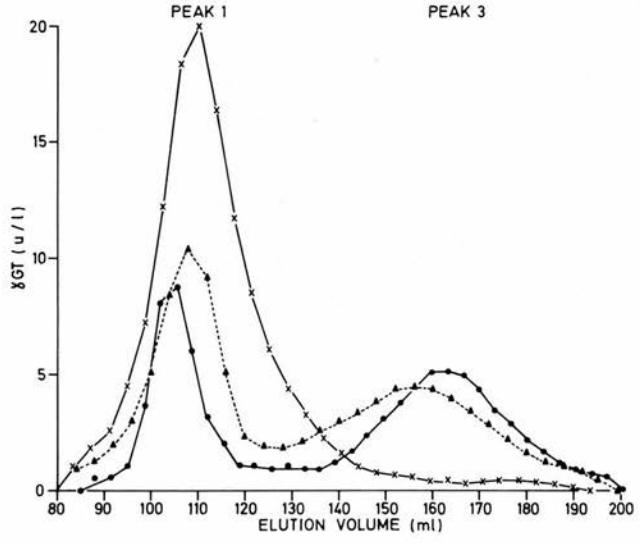

Fig. 2. Gel chromatography on Sephadex G200 of γGT in human bile with increasing concentrations of glycocholate in the elution buffer. ×———×, 5 mmol/l glycocholate; ▲------▲, 10 mmol/l glycocholate; ●———●, 20 mmol/l glycocholate.

the eluting buffer resulted in 90% of γGT, 84% of ALP and 71% of LAP eluting as Peaks 2T. Small amounts of activity (6–11%) still eluted as Peak 1, with the remainder as Peak 4.

Papain-treated bile

After papain treatment, gel chromatography in the absence of detergents resulted

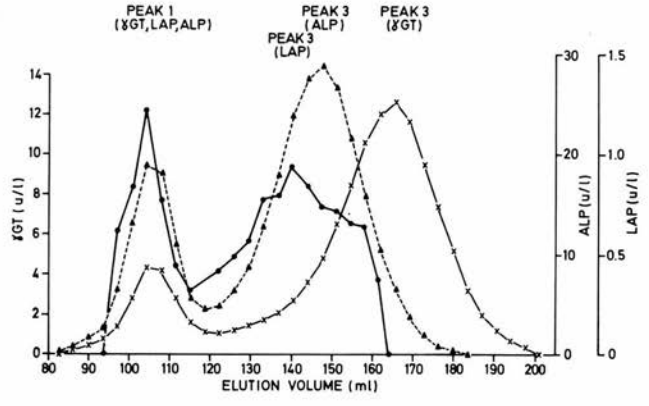

Fig. 3. Elution profiles of γGT, ALP and LAP following gel chromatography on Sephadex G200 containing 5 mmol/l glycochenodeoxycholate in the elution buffer. ×———×, γGT; ▲------▲, ALP; ●———●, LAP.

in a decrease in γGT and LAP activity eluting in the void volume, together with an increase in activity eluting as Peak 4. Since ALP activity was destroyed by papain a study of this enzyme was not possible.

7% Polyacrylamide gel electrophoresis

Electrophoresis in 7% polyacrylamide gel equilibrated in both the gel and the running buffer with 5 mmol/l glycochenodeoxycholate showed that both native bile and Peak 3 (γGT) contained a main zone of activity which migrated just ahead of the zone due to Peak 4. Electrophoresis of bile, Peak 3 (ALP) and Peak 4 (ALP) in the same concentration of bile salt, and stained for ALP activity, demonstrated a single fast band of identical mobility in all three samples. The Peak 4 (ALP) was obtained after gel chromatography of serum from a patient with liver disease. These results suggest that the Peak 3 forms of γGT and ALP possess a greater negative charge than the corresponding Peak 4 forms. Results for LAP could not be obtained owing to its inhibition by bile salts.

Gradient gel electrophoresis

The estimated M_r values for the Peak 4 forms of γGT and ALP in untreated bile were less than those for the corresponding Peak 3 forms (Table III) present in 5 mmol/l glycochenodeoxycholate.

Ultracentrifugation

Over 92% of the activity of all three enzymes was recovered from the supernatant following centrifugation at 2500 × *g*. After a further centrifugation of this supernatant at 150000 × *g* for 1 h, the percentage of enzyme activity recovered in the supernatant ranged from 25–73% for γGT, 22–48% for ALP and 27–54% for LAP. Addition of increasing concentrations of glycochenodeoxycholate to the bile prior to centrifugation resulted in more activity of all three enzymes being recovered in the

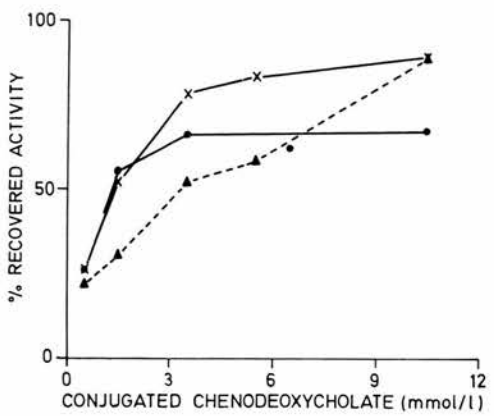

Fig. 4. Percentage recovery of enzyme activity in the supernatant following ultracentrifugation of bile at 150000 × *g*, plotted against initial conjugated chenodeoxycholate concentration. ×——×, γGT; ▲------▲, ALP; ●——●, LAP.

150000 × *g* supernatant. The increase in recovered supernatant activity was greater in those samples obtained from patients in whom bile had been draining for several days before the collection was made. They had a low concentration of bile salts due to depletion of the bile salt pool (Fig. 4).

Discussion

It has previously been assumed that the liver membrane-associated enzymes, γGT, ALP and LAP, are present in native bile as high M_r forms, possibly membrane fragments, since, after exclusion chromatography on Sephadex G200 or Sepharose 6B, these enzymes elute in the void volume [1–6]. However, we have shown that, if either glycocholate or glycochenodeoxycholate, at approximately physiological concentrations, or the non-ionic detergent Triton X-100 are included in the gel buffer, the elution patterns of the enzymes change markedly. The void volume peak contains less of the enzyme activity and lower M_r forms of the enzymes appear (Peak 3, Peak 2T). These lower M_r forms reaggregate if they are rechromatographed in detergent-free eluting buffer. The greater efficacy of glycochenodeoxycholate than glycocholate in causing this change can readily be explained in terms of the greater polarity of glycocholate.

The lower M_r forms of γGT, ALP and LAP which result from bile salt treatment (Peak 3 forms) have been shown here to be quite distinct in molecular size, charge and other properties from the low M_r forms found in untreated bile, or obtained following treatment with papain (Peak 4). The Peak 4 enzymes do not tend to aggregate, show no change of properties on the addition of bile salts, and have a significantly smaller M_r than the Peak 3 enzymes (Table III). By analogy with other membrane proteins, our results support previous suggestions [4,16] that the Peak 3 enzymes all contain a hydrophobic domain by which they are normally attached to the membrane; enzyme molecules possessing this hydrophobic region will tend to aggregate in aqueous solution unless an adequate concentration of detergent is present. Papain treatment, on the other hand, removes this hydrophobic portion of the molecule causing the difference in properties noted above. The three enzymes we have studied are very similar to one another in respect to their behaviour with bile salts and papain although the evidence is not quite complete since bile salts inhibit LAP activity and papain destroys ALP.

The ultracentrifugation studies show that between 25 and 75% of γGT, ALP and LAP activity in native bile is present in the 150000 × *g* supernatant. In samples in which the bile salt concentration is low, such as those obtained after several days of biliary drainage, the proportion of each of the enzymes present in the 150000 × *g* supernatant also tends to be low. We have obtained a similar effect in vitro by adding glycochenodeoxycholate to bile and thereby increasing the amount of enzyme present in the supernatant fraction. The simple interpretation of these findings is that the 150000 × *g* sediment contains particles or aggregated enzyme and that bile salts, whether endogenous or exogenous, will release the enzymes from these complexes.

At present there are two theories to account for the nature of the high M_r

enzymes in bile. One proposes that a low M_r enzyme is released into bile and forms aggregates with biliary lipid and protein [1,4]. The other suggests that the aggregates represent membrane fragments or small vesicles released by the hepatocyte or pinched off from the tips of the microvilli [17–19]. Our findings do not exclude either theory, nor do they exclude the possibility that both explanations might be true. However, it does seem that complexes studied in vitro, using aqueous media in which to separate and study the various forms, are very likely aggregates of the enzymes with lipids and proteins of the native bile, since it is the aqueous environment (i.e. without sufficient detergent action) that has caused the aggregation to occur.

The relationship between the biliary and serum enzymes remains obscure, as does the mechanism whereby serum enzyme activity rises in patients with cholestasis. Each of the enzymes is present in both serum and bile in both high and low M_r forms, the proportions of which vary depending on whether liver disease is present and, if present, on its nature [1–6,20]. The low M_r forms in serum in general resemble the enzymes which result from papain digestion of the enzymes in bile (Peak 4) and seem unlikely to originate from bile, which contains the Peak 3 enzyme but very little of the Peak 4 enzyme. We know too little about the high M_r forms of the enzymes present in bile and serum to speculate on their relationship to one another. One possibility, which fits most of the observed data, would be that in cholestasis there is induction of the membrane-bound enzymes in the liver and these are subsequently leached off the canalicular membrane by bile salts. An increased amount of biliary enzyme (Peak 3) might then find its way into plasma, where it would aggregate with lipids and possibly other proteins to yield a high M_r form.

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We would also like to thank Dr. G.J. Beckett for advice concerning the measurement of bile salts.

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Physical properties of γ-glutamyltransferase in human serum

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Summary

Using gel chromatography on Sephacryl S300 we have separated serum γ-glutamyltransferase into three fractions with estimated relative molecular masses of (a) greater than 1 000 000, (b) 250 000–500 000 and (c) about 120 000. Similarly, serum leucine aminopeptidase has been separated into three fractions. We have studied, particularly, the γ-glutamyltransferase fraction of intermediate relative molecular mass (250 000–500 000) in serum from patients with a number of liver diseases. We have shown, both by polyanion and immuno-precipitation, that it consists significantly of a complex between γ-glutamyltransferase and high density lipoprotein. The physical properties of this fraction, namely its mass and charge, can be altered by incubating serum with either bile or bile salts.

Introduction

Several studies have reported the presence of multiple forms of γ-glutamyltransferase (γGT, EC 2.3.2.2) in the sera of patients with liver disease [1–5]. Gel chromatography studies have confirmed the presence of three fractions of high, intermediate and low relative molecular mass (M_r) [6–10]. Two of these forms have been fairly well characterised. The form of high relative M_r is thought to consist of complexes in which hydrophobic γGT is associated with lipids, lipoprotein-X and other membrane enzymes, or membrane fragments [8,10–14]. In contrast, the low M_r form is hydrophilic and is thought to be produced by the action of neutral

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endopeptidases upon the hydrophobic enzyme either before or after its release into the circulation from the hepatobiliary tract. Only one detailed study, however, appears to have been made on the intermediate M_r forms with respect to their nature or physical properties [10]. This investigation suggested that such intermediate M_r forms consisted of complexes between γGT and high density lipoprotein (HDL).

The present study was undertaken to characterise the γ-glutamyltransferases, particularly those of intermediate M_r, and to compare their properties in patients with a wide variety of liver diseases. The objective was two-fold. Firstly, to ascertain whether any physical differences between the intermediate M_r forms in different patients might suggest the basis for a useful diagnostic test of liver disease. Secondly, to gain further insight as to possible mechanisms for the elevation of serum γGT activity in liver disease.

Two other enzymes present on the hepatocyte plasma membrane, aminopeptidase (microsomal) (leucine aminopeptidase, LAP, EC 3.4.11.2) and alkaline phosphatase (ALP, EC 3.1.3.1) were included in the study, where we considered appropriate to see if our findings were applicable on a wider basis to other membrane enzymes.

Materials and methods

Sera were obtained from 100 patients with liver disease and 10 apparently healthy individuals in whom there was no clinical or biochemical evidence of hepatic damage. The categories of liver disease included extrahepatic biliary obstruction, alcoholic cirrhosis, primary biliary cirrhosis, chronic active hepatitis, haemochromatosis, liver metastases and that due to anticonvulsant therapy.

Chemicals

Dextran sulphate ($M_r = 15\,000$) was obtained from Sochibo, Boulogne, France; antisera to human apolipoprotein A and apolipoprotein B from Hoechst, Hounslow, Middlesex, UK; Sephacryl S300 from Pharmacia, Uppsala, Sweden; γ-glutamyl-*p*-nitroanilide and *p*-nitrophenyl phosphate from Boehringer, Lewes, Sussex, UK; γ-L-glutamyl-α-naphthylamide and L-leucyl-β-naphthylamide from Koch Light, Colnbrook, Bucks, UK; sodium deoxycholate, sodium glycochenodeoxycholate, papain (twice crystallised), Fast Blue B, L-leucine-*p*-nitroanilide, from Sigma Chemical Company, Poole, Dorset, UK; acrylamide and bis-acrylamide from Eastman Kodak, Liverpool, UK. All other chemicals were obtained from BDH, Poole, Dorset, UK, and were of analytical grade.

Gel chromatography

This was performed on a 950 × 26 mm column of Sephacryl S300 equilibrated with 20 mmol/l Tris/HCl buffer (pH 8.0), containing 50 mmol/l sodium chloride. 3.5-ml fractions were collected with an upward flow rate of 28 ml/h. The column was calibrated for M_r determinations using a mixture of proteins of known M_r.

Polyanion precipitation

This was performed by the method of Burstein et al [15] using manganese chloride-dextran sulphate to precipitate low density lipoprotein (LDL) and very low

density lipoprotein (VLDL). After centrifugation, HDL was then precipitated by increasing the concentrations of $MnCl_2$-dextran sulphate. The lipoprotein precipitates were washed, then redissolved in a volume of 0.9% saline equal to the original volume of serum used and kept for further study.

Incubation of serum with lipoprotein antisera

10 μl serum was incubated with 50 μl antiserum to apolipoprotein A or apolipoprotein B or with 50 μl saline as a control, overnight at room temperature. 1 ml of ammonium sulphate (270 g/l) was then added and the mixture centrifuged at 11 000 × *g* for 10 min. γGT activity was determined on the supernatant.

For electrophoretic studies, 25 μl serum was incubated with 125 μl of antiserum overnight at room temperature. 25 μl of 40% sucrose containing 0.1% bromophenol blue was added to label the albumin, and 150 μl of the mixture subjected to electrophoresis.

General methods

7% polyacrylamide gel electrophoresis, polyacrylamide gradient gel electrophoresis, measurement of bile salt concentrations, localisation of enzyme activities after electrophoresis and measurement of enzyme activities were performed as previously described [16–22]. Reference ranges for serum from healthy adults were 6–31 U/l for females, 8–49 U/l for males for γGT; 27–70 U/l for LAP and 30–140 U/l for ALP.

Results

Treatment with papain

Papain digestion of serum had little effect upon γGT activity, but 34% of LAP activity and 43% of ALP activity was destroyed following papain treatment.

Gel chromatography

To agree with our previous arbitrary nomenclature [9,18], and simplify the description, we have numbered the peaks in order of their elution from the gel column (Figs. 1, 2). Quantitative differences in the relative sizes of the peaks were noted, but here we were concerned with their physical properties:

Peak 1: void volume for all enzymes.

Peak 2 (γGT), Peak 2 (LAP): major peak of intermediate M_r (Table I). For γGT, Peak 2 could take two forms (see below). No corresponding peak was present for ALP.

Peak 3 (γGT), Peak 3 (ALP): peaks of intermediate M_r present in the eluate only when bile salts are present in the eluting buffer. It is probable that the enzymes are present in bile in this form [18]. See Table I for estimated M_r values.

Peak 4 (γGT), Peak 4 (LAP), Peak 4 (ALP): peaks of low M_r (Table I) representing a small fraction of serum γGT but a major proportion of serum LAP and ALP activity. The size of this peak (for γGT and LAP) was increased greatly in sera that had previously been treated with papain.

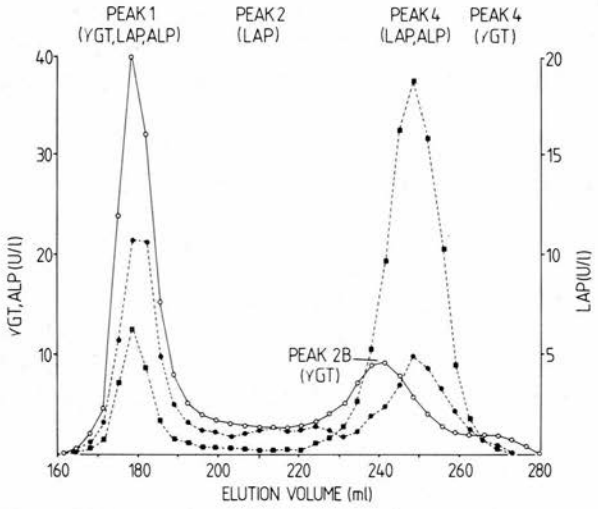

Fig. 1. Elution profiles of hepatocyte plasma membrane enzymes following gel chromatography on Sephacryl S300 of serum from a patient with extrahepatic biliary obstruction. ○——○, γGT; ●——●, LAP; ■——■, ALP.

Gel chromatography performed in the absence of bile salts

After gel chromatography of normal sera and sera from patients with liver disease, varying amounts of γGT and LAP eluted as Peaks 1, 2 and 4 (Table II). ALP on the other hand only appeared to exhibit activity in Peaks 1 and 4. Recovery of enzyme activity was always over 80%.

The elution profile of the second peak of γGT activity from most of the patients

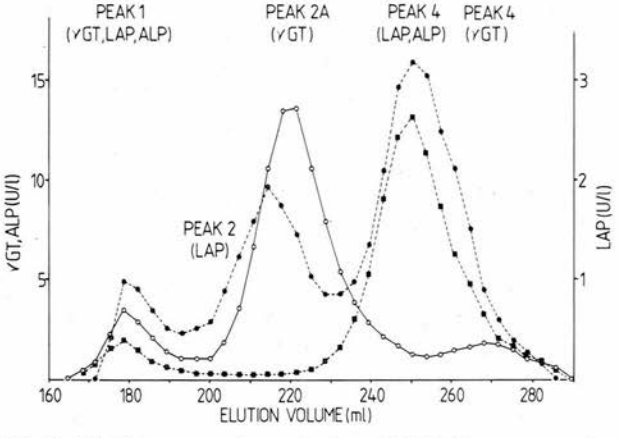

Fig. 2. Gel chromatography on Sephacryl S300 of hepatocyte plasma membrane enzymes in serum from a patient with alcoholic cirrhosis. ○——○, γGT; ●——●, LAP; ■——■, ALP.

TABLE I

Estimated molecular masses of the enzyme fractions obtained following gel chromatography

Fraction	Estimated molecular mass					
	gel chromatography			gradient electrophoresis		
	γGT	LAP	ALP	γGT	LAP	ALP
Peak 2A	325000–500000	–	–	300000–710000	–	–
Peak 2B	258000–295000	–	–	180000–220000	–	–
Peak 2	–	400000–610000	–	–	*	–
Peak 3	175000	–	280000	135000	–	260000
Peak 4	118000	190000	200000	98000	170000	180000

* The staining of this fraction appeared too faint and diffuse to make an accurate estimation of molecular mass.

with extrahepatic biliary obstruction (Peak 2B (γGT)) differed from that of most of the other patients and normal individuals (Peak 2A (γGT)) (Figs. 1, 2). Peak 2B (γGT) eluted later than Peak 2A (γGT) and was therefore of lower apparent M_r than Peak 2A (γGT). Of the 25 patients with extrahepatic biliary obstruction studied, 20 exhibited Peak 2B (γGT) activity only, three Peak 2A (γGT) activity only, one approximately equal amounts of both and one neither peak activity.

All possessed variable amounts of Peaks 1 and 4 (γGT). It was noted that in some jaundiced samples from patients with other pathologies, a shoulder was observed on Peak 2A (γGT) in the position of Peak 2B (γGT). Analogous results were obtained for LAP (Figs. 1, 2) except that Peak 2 (LAP) did not vary so much in its elution profile, and appeared to constitute a smaller proportion of total activity in those patients with obstructive jaundice compared to the rest. In contrast to both γGT and LAP, ALP showed no intermediate M_r peaks (Figs. 1, 2).

Gel chromatography in the presence of bile salts

The fractions containing Peaks 1 and 2 obtained from gel chromatography were

TABLE II

Distribution of hepatocyte plasma membrane enzymes obtained after gel chromatography

Fraction	Percentage of total recovered activity	
	mean	range
Peak 1 (γGT)	27.3	6–82
Peak 2 (γGT)	57.4	0–83
Peak 4 (γGT)	15.3	2–58
Peak 1 (LAP)	18.5	3–60
Peak 2 (LAP)	22.5	0–53
Peak 4 (LAP)	59.0	27–91
Peak 1 (ALP)	14.0	2–54
Peak 4 (ALP)	86.0	46–98

pooled, concentrated by ultrafiltration and rechromatographed in the presence of either 12 mmol/l glycochenodeoxycholate or 12 mmol/l deoxycholate. Following rechromatography, Peak 1 (γGT) and Peak 2 (γGT) disappeared, giving rise to Peak 3 (γGT). Peak 1 (ALP) similarly disappeared, giving rise to Peak 3 (ALP). No LAP activity was recovered in the column eluate.

Papain-treated sera

Gel chromatography of papain-treated sera in the absence of bile salts resulted in a decrease in γGT and LAP activity eluting as Peaks 1 and 2, together with an increase in activity eluting as Peak 4. There was also a decrease in activity of Peak 1 (ALP), but no concomitant increase in activity associated with Peak 4.

Electrophoresis

Electrophoresis on 7% polyacrylamide gel showed two fairly distinct patterns of γGT activity, according to patient group. In sera from patients with extrahepatic biliary obstruction, two main bands of activity were seen, one at the origin (Band I (γGT)), together with another of mobility 45–55% of albumin (Band IIB (γGT) (Fig. 3)). Occasionally, smaller, minor zones of slower mobility were seen. The sera from other groups of patients and the normal sera showed a smaller zone of activity at the origin, with significant activity occurring in various zones (Bands IIA (γGT) (Fig. 3)), with mobilities between 8 and 40% of that of albumin.

Minor zones were often present, corresponding to Band IIB (γGT) in abnormal but not in normal sera. Sera from all patients showed a less intense zone of activity (Band IV (γGT) (Fig. 3)), of mobility between 70 and 80% of that of albumin. This zone was also present in normal sera and seemed to contribute a greater proportion of total activity. Electrophoresis of concentrated pooled fractions corresponding to Peaks 2A (γGT) and 2B (γGT) obtained after gel chromatography revealed that

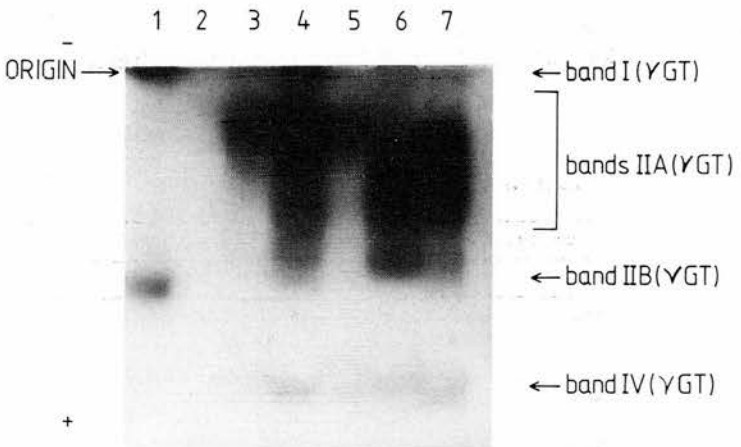

Fig. 3. 7% polyacrylamide gel electrophoresis of γGT in sera from patients with liver disease. 1, extrahepatic biliary obstruction; 2, blank; 3–7, alcoholic cirrhosis.

Peak 2A (γGT) was heterogeneous, giving rise to several zones (Bands IIA (γGT)). Peak 2B (γGT) on the other hand gave rise to an electrophoretically discrete band (Band IIB (γGT)). Similarly, Band IV (γGT) was shown to have identical mobility to concentrated pooled fractions corresponding to Peak 4 (γGT).

Analogous results were obtained for LAP. However, the bands of intermediate mobility (between 5 and 35% of that of albumin) did not appear to display any disease-specific distribution. The fraction corresponding to Peak 4 (LAP) obtained from gel chromatography gave rise to two bands with mobility approximately 55 and 60% of that of albumin (Band IVA (LAP) and Band IVB (LAP)). Electrophoresis of papain-treated sera yielded a single band of γGT activity with mobility identical to that of Peak 4 (γGT). Similarly, a single band of LAP activity of equal mobility to Band IVB (LAP) was obtained.

Polyacrylamide gradient gel electrophoresis

This confirmed the heterogeneity of Peak 2 for γGT (Table I).

Polyanion precipitation

Gel chromatography was performed on the redissolved lipoprotein fractions of LDL and VLDL, and HDL. Between 40 and 75% of Peak 1 (γGT) activity co-precipitated with the LDL and VLDL fraction, together with a much smaller proportion of Peak 2A (γGT) (Fig. 4). Between 49 and 65% of Peak 2A (γGT) was precipitated with the HDL fraction, whereas Peak 2B (γGT) did not co-precipitate with any of the lipoprotein fractions.

These results with Peak 2A (γGT) provide circumstantial evidence that this γGT

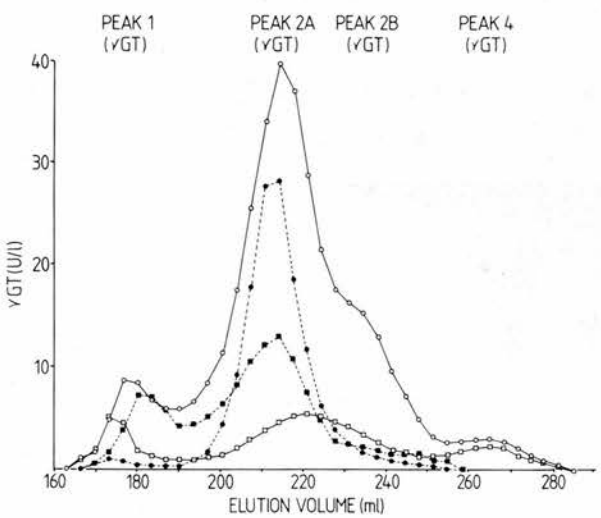

Fig. 4. Elution profile of γGT in serum and the lipoprotein fractions from a patient with alcoholic liver disease before and after precipitation with polyanions. ○———○, whole serum; ■———■, LDL, VLDL; ●———●, HDL; □———□, supernatant after precipitation of LDL, VLDL and HDL.

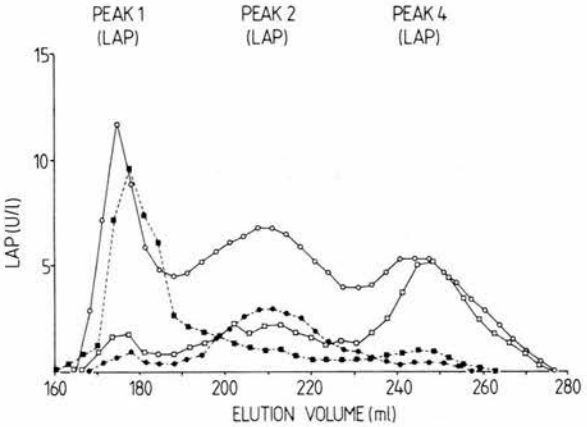

Fig. 5. Elution profile of LAP in serum from a patient with gallstones before and after precipitation of the lipoprotein fractions with polyanions. ○———○, whole serum; ■———■, LDL, VLDL; ●———●, HDL; □———□, supernatant after precipitation of LDL, VLDL and HDL.

fraction is a part of HDL. Although it is possible that the polyanion precipitation procedure coincidentally precipitates γGT protein, this was demonstrated to be unlikely since: (1) polyanion precipitation of serum did not appreciably alter the total recovered protein in the supernatant; and (2) the addition of polyanions to Peak IV (γGT) diluted in serum (from which HDL had been removed, and using the polyanion conditions suitable for HDL precipitation) did not precipitate any γGT.

Analogous results were obtained for LAP, with Peak 1 (LAP) co-precipitating with LDL and VLDL, and Peak 2 (LAP) co-precipitating with HDL (Fig. 5). ALP activity, however, only precipitated with the VLDL and LDL fractions.

Incubation with antisera

Sera from 69 patients were incubated with antiserum to apolipoprotein A; this

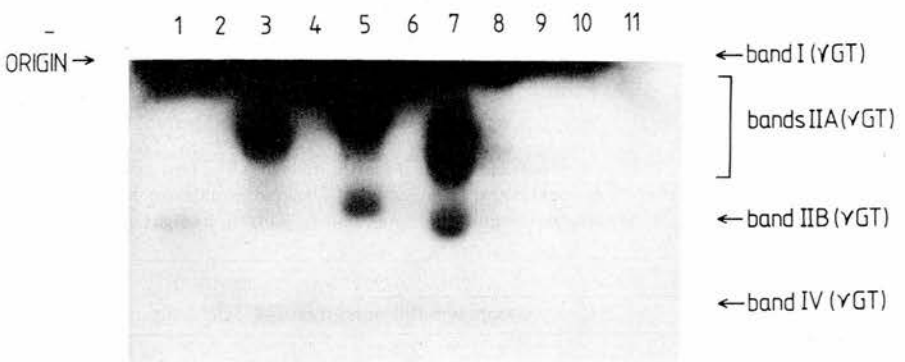

Fig. 6. Polyacrylamide slab gel electrophoresis of sera from patients with liver disease before and after incubation with antiserum to apolipoprotein A. 1, 3, 5, 7, 9, before; 2, 4, 6, 8, 10, after; 11, antiserum to apolipoprotein A.

caused precipitation of a variable amount of γGT activity (mean 59.9%, range 8–92%). On the other hand, no γGT was precipitated either after incubation of the sera with antiserum to apolipoprotein B, or when Peak 4 (γGT) was incubated with antiserum to apolipoprotein A.

Electrophoresis of the mixtures of serum with apolipoprotein A antiserum resulted in a marked decrease in staining intensity of the bands of intermediate mobility (Fig. 6). Inspection of the gels also suggested an increase in intensity of staining at the origin for both γGT and LAP.

Immunoelectrophoresis of the LDL and VLDL fraction obtained by polyanion precipitation, and of HDL-free serum against antiserum to apolipoprotein A did not reveal the presence of any precipitin arcs when stained for protein. On the other hand, immunoelectrophoresis of the HDL fraction against antiserum to apolipoprotein A gave rise to a single precipitin arc in the α_1-globulin region.

Incubation of sera with bile

Sera from patients that possessed Peak 2A (γGT) activity were incubated with an equal volume of hepatic bile for 3h at 37 °C. The mixture was then subjected to gel chromatography or electrophoresis.

Gel chromatography of the mixture revealed that the Peak 2A (γGT) activity appeared to change to Peak 2B (γGT) activity (Fig. 7). Occasionally this change was associated with an apparent increase in total activity recovered from the sera/bile mixtures, possibly due to a matrix effect of the serum proteins on biliary γGT.

Electrophoresis of the mixture resulted in a marked decrease in intensity of Bands IIA (γGT) and the appearance of a zone (Band IIC (γGT)) ahead of Band IIB (γGT) (Fig. 8). Similarly, electrophoresis of bile-treated sera from patients with obstructive jaundice possessing Band IIB activity resulted in the appearance of Band IIC (γGT). Usually this was associated with a decrease in the intensity of staining of Band IIB (γGT).

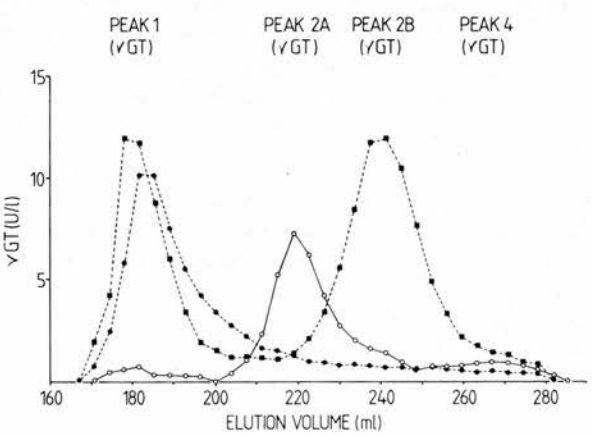

Fig. 7. Elution profile of γGT in the serum of a patient with alcoholic cirrhosis before and after incubation with hepatic bile. ○——○, serum before; ■——■, serum and bile; ●——●, hepatic bile.

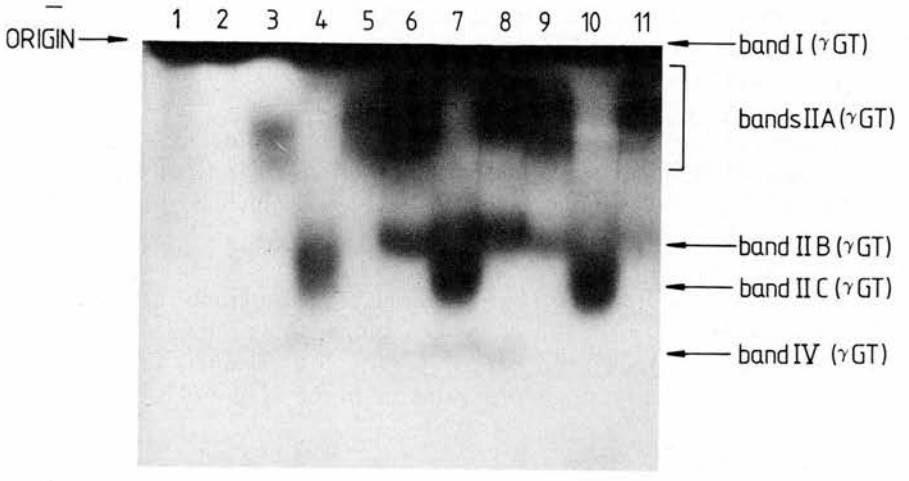

Fig. 8. Polyacrylamide slab gel electrophoresis of sera from patients with liver disease before and after incubation with dialysed or undialysed hepatic bile. 1, undialysed hepatic bile; 2, dialysed hepatic bile; 3, 6, 9, sera + saline; 4, 7, 10, sera + undialysed bile; 5, 8, 11, sera + dialysed bile.

Incubation of the various bile-sera mixtures with antiserum to apolipoprotein A, followed by electrophoresis, resulted in a decrease in intensity of staining of Band IIC (γGT) as compared to mixtures that had been incubated with a saline control.

The ability of bile to effect these changes on Peak 2A (γGT) and Bands IIA (γGT) and IIB (γGT) was removed by prior dialysis of the bile against 20 mmol/l Tris/HCl buffer (pH 8.0) (Fig. 8).

Incubation of sera with bile salts

Patients' sera were incubated with an equal volume of glycochenodeoxycholate at concentrations ranging from 10 μmol/l to 20 mmol/l for 3 h at 37 °C. Electrophoresis of the mixtures in either 7% polyacrylamide or 4–30% polyacrylamide gradient gels showed that incubation with glycochenodeoxycholate at a concentration greater than, or equal to 6 mmol/l resulted in a decrease in intensity of Bands IIA (γGT) and IIB (γGT). This phenomenon appeared to be independent of the initial concentration of total conjugated chenodeoxycholate in the sera studied. For the patients with extrahepatic biliary obstruction this was: mean 64 μmol/l; range 9–229 μmol/l. For the others the mean was 12 μmol/l; range 0–113 μmol/l.

A sample of serum from an alcoholic patient containing Peak 2A (γGT) activity was incubated with an equal volume of 20 mmol/l glycochenodeoxycholate for 3 h at 37 °C. Gel chromatography of the mixture revealed a shift in the elution profile of Peak 2A (γGT) to that approximating to Peak 2B (γGT). No change in the elution profile of Peak 1 (γGT) was observed.

Effect of dialysis and freezing and thawing

Dialysis against 20 mmol/l Tris-HCl (pH 8.0), or freezing to −60 °C, followed by thawing at 37 °C, had no effect either upon the elution patterns of the enzymes from the Sephacryl column or on their electrophoretic mobility.

Studies on Peak 3 (γGT)

Gel Chromatography was performed on a patient's serum containing Peaks 2A (γGT) and 2B (γGT), in the presence of 12 mmol/l deoxycholate and the Peak 3 (γGT) obtained was pooled, concentrated and dialysed against 20 mmol/l Tris/HCl (pH 8.0). Peaks 2A (γGT) and 2B (γGT) were also obtained by performing gel chromatography in the absence of bile salts and a single concentrated pool obtained.

The original serum, and each of the two concentrated pools (Peak 3 (γGT)), and Peaks 2A (γGT) and 2B (γGT) were then incubated overnight at room temperature with an equal volume of antiserum to apolipoprotein A and saline as a control. The mixtures were then subjected to electrophoresis and stained for γGT activity. A decrease, compared to a saline control, in enzyme staining in the areas corresponding to Bands IIA (γGT) and IIB (γGT) was observed in the original serum and the Peaks 2A (γGT) and 2B (γGT) pool after incubation with antiserum. No such decrease in intensity of staining was observed in the sample representing Peak 3 (γGT).

The two pools representing the combined Peaks 2A (γGT) and 2B (γGT) and Peak 3 (γGT) were then incubated overnight at room temperature with an equal volume of a normal serum prior to incubation with antiserum, and subjected to electrophoresis. On staining for γGT activity, a decrease in staining intensity of the zones of intermediate mobility was observed in all of the incubations with antiserum as compared to a saline control.

Discussion

Our present study confirms previous reports [6–11,23] that γGT is present in normal sera and in sera from patients with liver disease in high, intermediate and low M_r forms. We have also shown that another hepatocyte plasma membrane enzyme, LAP, is present in high, intermediate and low M_r forms, unlike ALP which does not possess the intermediate M_r form.

The high M_r forms of all three enzymes co-precipitated with VLDL and LDL after treatment with polyanions. This is consistent with previous reports that they may consist of complexes of the enzymes with lipoproteins or lipoprotein-X [8,10,12,24,25].

In contrast, varying amounts of the intermediate M_r enzymes with the notable exception of Peak 2B (γGT), co-precipitated with HDL, confirming the findings of Huseby [10]. On the other hand, all of the intermediate M_r enzymes including Peak 2B (γGT), were shown to bind to antiserum to apolipoprotein A but not to antiserum to apolipoprotein B. Taken together, these findings strongly suggest that the intermediate M_r forms of both γGT and LAP consist of complexes of the enzymes with HDL. Their estimated molecular mass (Table I) is consistent with the attachment of a single molecule of γGT or LAP onto an HDL particle whose molecular mass may range from 180 000 to 350 000 [10]. However, the wide range of molecular mass, particularly in Peak 2A (γGT), does not preclude the attachment of more than one enzyme molecule per HDL particle. Up to 30% of the intermediate M_r enzymes also co-precipitated with LDL and VLDL (Figs. 4, 5). This suggests

either that the presence of the intermediate M_r enzymes on the HDL complexes may have altered the behaviour of some of the complexes with polyanions or that the enzymes may have been associated with LDL or VLDL. The latter possibility is rendered unlikely by the fact that these intermediate M_r enzymes were not precipitated by antiserum to apolipoprotein B. The reason for Peak 2B (γGT) not precipitating with polyanions is unclear to us. It is possible, although we have no evidence of this, that the presence of high concentrations of bile salts in the serum of these patients may have altered the precipitation characteristics of these particles.

The mean bile salt concentration in the serum of patients with obstructive jaundice were over five times those of the other groups. Consequently, in an attempt to mimic the serum abnormalities found in obstructive jaundice, sera containing Peak 2A (γGT) were incubated with hepatic bile. After incubation Peak 2A (γGT) was converted to a peak with an elution pattern similar to that of Peak 2B (γGT). The ability of bile to effect this change was (a) removed by prior dialysis of the bile, and (b) reproduced by incubation with glycochenodeoxycholate. These results suggest that the conversion of Peak 2A (γGT) to Peak 2B (γGT) was caused by the bile salts present in the bile. Electrophoresis of the bile/bile salt/sera mixtures however, showed the presence of Band IIC (γGT), not previously detected in any sera. This would suggest that factors, other than bile salt concentrations, are involved in the formation of Peak 2B (γGT) in the sera of patients with obstructive jaundice. This is supported by the fact that dialysis appeared to have no effect upon Peak 2B (γGT) in terms of its M_r or total recovered activity.

When gel chromatography was performed in the presence of bile salts, the Peak 3 forms of the enzymes were obtained. These peaks are identical to those already described in hepatic bile and sera [9,11,18,23,25], although the picture is incomplete because bile salts inhibited serum LAP activity. Unlike the hydrophilic Peak 4 forms, observed in untreated sera, or obtained following treatment with papain, the Peak 3 forms were hydrophobic in nature and readily re-aggregated if the bile salts were removed by dilution or dialysis.

The inability of antiserum to apolipoprotein A to bind Peak 3 (γGT) suggests that the γGT-HDL complex had either been dispersed or radically altered in its properties. In an attempt to reform the complex, Peak 3 (γGT) was incubated with normal serum, after which the antiserum appeared able to bind to the γGT in the serum-Peak 3 (γGT) mixture. These findings imply that the γGT-HDL interaction is hydrophobic in nature and that it can form spontaneously in serum in vitro. The complex also appears to have a stable physical configuration since freezing and thawing had no noticeable effect.

The mechanism whereby serum γGT activity rises in patients with liver disease remains obscure. The serum enzyme activity is elevated to varying extents in many disease states. This elevation appears to be due to the presence of so many different physico-chemical forms [1–10] that it is difficult to envisage a single unifying hypothesis that would explain all of the observed elevations. What is probable is that the Peak 3 (γGT) described here, and previously proposed [18] to be the major component of γGT in bile (where there is a high concentration of bile salts), is in some way involved. This molecule is hydrophobic in nature and readily re-aggregates

in the absence of bile salts. Biliary obstruction, liver cell damage, alcohol excess or drug therapy may cause induction of this enzyme form, which may be leached off the plasma membrane by bile salts, and subsequently released into the circulation. Once in the circulation, and consequently in an environment of lower bile salt concentration, the enzyme would be free to re-aggregate with itself or to form complexes with one or other of the lipoprotein fractions present in the plasma. The lipoprotein fractions to which it complexes would depend upon the disease process which is occurring. However, the factors which dictate the particular lipoprotein fractions with which the enzyme associates remain unknown.

Finally, our finding that Peak 2B (γGT) is found in large amounts only in patients suffering from extrahepatic obstructive jaundice suggests that measurement of this fraction might be of clinical and diagnostic value.

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